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THE INTERACTION OF BLOOD PLATELETS WITH BIOGENIC AMINES: INHIBITORY EFFECTS OF HALOPERIDOL, IMIPRAMINE AND NH_4Cl ON THE UPTAKE OF DOPAMINE AND SEROTONIN.

University — Université

UNIVERSITY OF ALBERTA

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

MASTER OF SCIENCE

Year this degree conferred — Année d'obtention de ce grade	Name of Supervisor — Nom du directeur de thèse
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THE INTERACTION OF BLOOD PLATELETS WITH BIOGENIC AMINES: INHIBITORY EFFECTS OF HALOPERIDOL, IMIPRAMINE AND NH_4Cl on THE UPTAKE OF DOPAMINE AND SEROTONIN.

by

Michael W. Stewart

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

EXPERIMENTAL MEDICINE

DEPARTMENT OF MEDICINE

EDMONTON, ALBERTA

FALL, 1982

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DEGREE FOR WHICH THESIS WAS PRESENTED MASTER OF SCIENCE

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.....

Supervisor

.....

.....

Date October 18/82

DEDICATION

To my wonderful wife Laurie, whose constant support, understanding and love inspired the completion of this degree and to our future child.

ABSTRACT

The uptake of dopamine and serotonin was monitored in column-washed blood platelets. The uptake of both biogenic amines increased with exogenous ligand concentration showing no sign of saturation. Haloperidol, imipramine and NH_4Cl were tested as uptake inhibitors. Dopamine and serotonin presented an ammonium chloride sensitive component of accumulation. This appeared to saturate at 0.1 mM for the uptake of dopamine. Imipramine proved to be a more potent inhibitor of serotonin uptake while haloperidol was more effective in inhibiting dopamine accumulation. A low affinity dopamine binding site was identified on crude platelet membrane preparation. This apparent receptor exhibited a K_D of 184 nM with 39 fmols/mg protein binding sites. The evidence presented is indicative of a carrier mediated transport process for dopamine unique from that of serotonin.

ACKNOWLEDGEMENTS

I would like to convey my deepest appreciation to Dr. Taichi Nihei, whose calm level-headed approach to problems, unparalleled knack for conveying complex ideas and friendship proved invaluable in my mental and emotional growth over the past two years.

My thanks to Carol, Cindy, Sandy and Chris from rheumatology for their expert direction in venipuncture and willingness to lend a friendly ear when Murphy's laws determined the outcome of an experiment.

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CHAPTER I

INTRODUCTION

Blood platelets accumulate dopamine (DA) against a concentration gradient in a manner similar to the uptake of 5-HT (Solomon et al, 1970; Gordon and Olverman, 1978). This DA uptake is described previously (Sneddon, 1973; Gordon and Olverman, 1978) to be mediated by the carrier responsible for 5-HT uptake. Kinetic studies (Gordon et al, 1977; Gordon and Olverman, 1978) have shown that DA and 5-HT are mutually antagonistic in the uptake process in blood platelets. The inhibitory constant of one amine for the uptake of the other was shown to approximate its Km value for uptake. This supports the possibility that DA and 5-HT share the same carrier. In an attempt to characterize the relationship between the uptake of DA and 5-HT various inhibitor of amine accumulation were employed (Trenchard et al, 1975; Airaksinen et al, 1980). Some inhibitors proved to be more effective on 5-HT uptake while others were more effective on DA uptake. This supports the possibility of a separate mechanism for the accumulation of DA by blood platelets.

The uptake of DA (Gordon and Olverman, 1978) and 5-HT (Sneddon, 1973) can be divided into two components, a saturable energy-dependent process and a diffusion process. The identification of these two components is difficult. Gordon et al (1977) indicated that short incubation periods (< 10 seconds) at low 5-HT concentration (< 10 μM) in the incubation medium would allow the energy-dependent process to predominate. These authors also recognized that even at DA concentrations

below the saturation level of uptake for the energy-dependent process, diffusion accounted for a significant amount of uptake.

5-HT and DA uptake by blood platelets are inhibited by various compounds such as tricyclic antidepressants (Trenchard et al, 1975; Rudnick, 1977), metabolic poisons (Solomon, 1970; Gordon et al, 1977) and β-carbolines (Airaksinen et al, 1980). Imipramine (Rudnick et al, 1980) inhibits the Na^+ dependent uptake (Sneddon, 1973) of 5-HT in platelet plasma membrane vesicles while reserpine inhibits the ATPase dependent accumulation of 5-HT in isolated platelet dense granules (Rudnick, 1980). Haloperidol (haldol), a DA antagonist in the brain (Creese et al, 1981) inhibits the uptake of DA by blood platelets (Solomon et al, 1970). Proton ionophores such as carbonyl cyanide p-triflouromethoxyphenylhydrazone (FCCP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) collapse existing pH gradients across isolated dense granules and cause efflux of accumulated 5-HT (Carty et al, 1981). DA has been shown to accumulate in liposomes in response to artificially imposed pH gradients - interior acidic (Nichols and Deamer, 1976). Addition of 10 mM NH_4Cl collapses the pH gradient resulting in efflux of accumulated DA. Similar results were obtained by Ingebretsen and Flatmark (1978) who studied the uptake of DA by isolated bovine adrenal chromaffin granules.

A DA binding site on the platelet membrane has not been identified. Estimated values of the affinity constant of DA binding to platelet membranes based on inhibition of agonist binding (Cheng and Prusoff, 1973) range from 3.1 μM (Newman et al, 1978) to 19 μM (Lynch and Steer, 1981). Such variation of binding constant estimates have

been suggested to be due to a low density of binding sites (Tsai and Lefkowitz, 1978).

It thus seemed of value to determine whether DA and 5-HT were accumulated in blood platelets by similar mechanisms. Amine uptake inhibitors (haldol, imipramine and NH_4Cl) were employed to characterize their effect on DA and 5-HT uptake in an attempt to differentiate between the two processes. If DA uptake is mediated by a carrier separate from the one responsible for 5-HT accumulation, then the possibility of a DA binding site to the platelet membrane exists. Binding studies were carried out on crude platelet membrane preparations to determine whether or not a DA binding site exists.

CHAPTER II

REVIEW OF LITERATURE

A. PLATELET FUNCTION AND MORPHOLOGY

The platelet is a membrane bound structure essential in haemostasis. Although variable in shape, it is usually discoid. The average diameter is 2 - 4 μm^3 (Karpatkin, 1977). Anucleate, the platelet has no means of replication although it contains many of the structures found in a normal cell. There is very little endoplasmic reticulum or golgi apparatus. A hazy coat composed of carbohydrate or glycoprotein 10 - 20 nm thick surrounds the trilaminar platelet membrane which is 7 9 nm thick. The outside coat has receptors for the following factors of the coagulation system: I, V, VII, XI, XII and XIII. The platelet membrane also has receptors for monamines (eg. serotonin, dopamine, epinephrine), ADP (important in platelet aggregation), thrombin and glycosyl transferase.

Within the platelet membrane, two types of granular components are observed; the alpha granules and the dense granules. The alpha granules are more numerous than the dense granules and are associated with lysosomes. The dense granules are a source of non-metabolic ADP, an important component in the sequence of events leading to platelet aggregation and subsequent haemostasis. They also contain serotonin and small amounts of catecholamines. Interestingly enough, these granules also store calcium (Johnson, 1971).

A myriad of tubules, known as the surface connecting system, course through the platelet and are thought to increase the effective surface area of the platelet for excretion (Triplett, 1968).

Mitochondria, necessary in energy production, are seen scattered throughout the cytoplasm. They are closely associated with the microtubular system responsible for maintenance of cell shape. Contact of the platelet with a hydrophilic surface causes characteristic changes in structure. Surfaces causing this response are collagen (exposed due to damage to the endothelial lining of a vessel) and glass. The platelet, upon contact with collagen for example, begins to swell and assume irregular forms. Pseudopodia are seen projecting haphazardly from the body of the platelet. The surface of the platelet becomes sticky and it adheres to the exposed collagen. ADP, stored within the dense granules, is secreted in large amounts and activates nearby platelets. As more and more platelets become sticky, they coalesce about the area of damage in the vascular endothelium, thus forming a platelet plug. Thrombin, a constituent of blood plasma, then acts on the aggregated platelets causing them to form a tight and unyielding plug.

Platelets are necessary for clot retraction. Not only do platelets contain the contractile elements but also contain large amounts of ATP. Thus, when the clot retracts, expressing the serum, the edges of the rent in the vessel wall are drawn together and the process of repair takes place.

Platelets are produced by megakaryocytes, inhabitants of the bone marrow. The production of megakaryocytes can be stimulated by

thrombocytopenia, a decrease in the number of circulating thrombocytes (platelets). Studies have shown the life span of the platelet in the blood stream to be 7 - 10 days (Johnson, 1971).

After their release from the bone marrow, platelets apparently do not enter a pool in which they are evenly distributed through the circulation (Johnson, 1971). The spleen appears to be the major area in which platelet concentration exceeds that of the peripheral blood.

The following text obtained from The Circulating Platelet (S. Johnson, 1971) provides a historical review of essential observations of the morphological, physical and chemical characteristics of platelets:

> In the mid 1800's, with the advent of the achromatic objective to negate chromatic aberration, Guliver, Addison and Simon viewed the tiny and yet unamed membrane bound structures. Zimmerman, in 1846, reported 'small colorless bodies, refractile and with well defined outlines."
>
> It was not until 1865, when Shultze observed platelet aggregation using a heated stage, that anyone had an inkling that platelets might play some role in the maintenance of haemostasis. Osler, in 1874, showed aggregation occurred outside the vessel. He also described platelet pseudopodia as "two, three or even more taillike processes of extreme delicacy". A year earlier, Ranvier and Vulpian noted that fibrin formed in relation to granules in the blood and that these granules stuck to glass.
>
> Separation of platelets from the blood was accomplished by Bizzozero in 1882. In so doing, he was able to determine the time for re-establishment of normal numbers. It was Bizzozero who coined the term 'platelets'.
>
> In 1896, Haymen, working off the findings of Krauss (1883) and Denys (1887), related clot retraction to the presence of platelets.
>
> Megakaryocytes were indicated to be the origin of platelets in 1906. Wright observed that the megakaryocyte cytoplasm gave rise to the platelet by

"pinching off small rounded projections or pseudopods from the cell body". Cleavage was noted to take place through a zone of hyaline encircling the future platelet.

A wave of technological advances allowed Wolpers and Ruska to view the platelet under the electron microscope in 1939.

Further electron microscopic work was done by Bessis and Burnstein from 1948 - 1950. These researchers described the structural states of the platelet as follows: 1)discoidal or round, 2) dendritic, 3)transitional, 4)expanded or spread.

Rebuck et al (1960, 1961) modified the above scheme as follows: 1)circulating or round forms, 2)dendritic or pseudopodial stages, leading to, 3)platelet aggregation or early viscous metamorphisis, 4)intermediate or transitional, 5)expanded or spread forms.

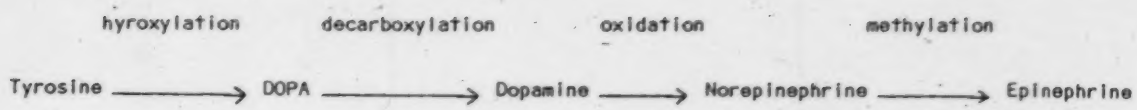
A year earlier, Bessis and Pulvertaft observed direct formation of platelets from megakaryocytes. Around the same time Johnson confirmed lipid production by the dense azurophilic granules ellucidated by Wright in 1906.

Born, in 1956, discovered large amounts of ATP in the platelet and posulated that ADP, a breakdown product of ATP hydrolysis, was influential in aggregation. This fact was born out by Hellum (1960) and Guarder (1961). Hovig (1962), Rodman (1963) and Zucker (1964) linked the presence of ADP with increased stickiness of the hazy coat of the platelet, which predisposed aggregation.

The microtubular element of the platelet was discovered by White in 1967. He surmized that they were influential in the maintenance of the platelets' discoid shape. The trilaminar cell membrane of the platelet was found by Behnke (1968). With this discovery, the possiblity existed that a semipereable membrane enveloped the platelet.

B. DOPAMINE

The production of catecholamines in the brain starts with the amino acid tyrosine as a precursor. A series of hydroxylation and decarboxylation steps results in the generation of dopamine (DA).



Greater than 50 % of the total brain catecholamines are in the form of dopamine. The neostriatum, nucleus accumbens and tuberculum olfactorium are regions of high DA concentration. The superior cervical ganglion also contains large amounts of DA which is thought to be located in small intensely flourescent cells.

DA appears to govern motor function in the nigrostriatal region (Cooper et al, 1974) and hypothalamic-pituitary control in the tuberoinfundibular system. It is postualted that DA may play some role in mental function in the mesolimbic system. Parkinson's disease is associated biochemically with a deficiency of DA in the dopaminergic neurons of the caudate, putamen, globus pallidus and substantia nigra. The disease presents the following symptoms: tremor of the hands, face and tongue; akinesia; and intermittent rigidity (Robbins and Angell, 1976).

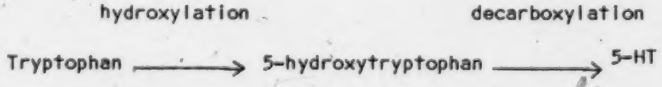
There is evidence that DA is present in discrete nerve endings (Guyton, 1977) and is stored within subcellular granules. Activation by a nerve impulse causes the release of DA from the storage granules into the synaptic cleft. DA then acts on a receptor which initiates post-synaptic events. Several drugs are known to interfere with this process of dopaminergic nerve transmission. Reserpine interferes with the uptake/storage mechanism of amines in the amine granules. DA receptors can be stimulated by drugs such as apomorphine and dihydroergocryptine (Cooper et al, 1974). DA receptors are inhibited by drugs such as haloperidol (Creese et al, 1981).

In the brain, DA is taken-up into the presynaptic terminal after excitation; thus terminating its action (Cooper et al, 1974). The peripheral nervous system also displays DA production and breakdown. Most of DA is oxidized to norepinephrine (NE), and the rest is deaminated by monoamine oxidase (MAO) (Cooper and Roth, 1974).

C. SEROTONIN

Serotonin (5-HT) is located in several cell systems of the body. We find the highest concentration of 5-HT per unit cell mass in the pineal gland and enterochromaffin cells of the intestinal tract. Approximately 10% of the total body 5-HT is found in the blood platelets. The 5-HT found in blood platelets is concentrated in osmiophilic granules. Only 1 - 2% of the total body 5-HT is situated in the central nervous sytem (CNS).

The synthesis of 5-HT in the brain occurs via the following pathway:



Monamine oxidase (MAO) may deaminate 5-HT after it has been synthesized. This catabolic degradation results in the production of 5-hydroxyindoleacetaldehyde. This in turn may be oxidized to 5-hydroxyindoleacetic acid (5-HIAA) or reduced to 5-hydroxytryptophol.

The use of a flourescense technique pioneered by Hillarp and Falk (1968), indicated that almost all 5-HT containing cell bodies were found in the raphe nuclei.

5-HT is accumulated by blood platelets (Sneddon, 1973) and neurons (Cooper and Roth, 1974) by a carrier mediated process that is energy dependent, sodium ion dependent, and requires glucose and oxygen. This uptake can be inhibited by metabolic poisons such as ouabain, dinitrophenol and iodoacetate (Cooper and Roth, 1974). The uptake of 5-HT by blood platelets and a number of other neurotransmitters is also inhibited by drugs such as imipramine (Rudnick et al, 1980), protriptyline (Trenchard et al, 1975) and β-carbolines (Airaksinen et al, 1980).

Although knowledge of the physiological role of 5-HT is presently incomplete, some experiments (Cooper and Roth, 1974) have demonstrated the role of 5-HT in some parts of the central nervous system (CNS). By injecting 5-HT directly into the brain ventricles, researchers discovered that this monoamine elevates body temperature. 5-HT has also been associated with the ability to perceive and react to sensory stimuli. In addition, sleep duration is affected by the presence or absence of 5-HT (Cooper and Roth, 1974).

Q.- UPTAKE OF DOPAMINE (DA) AND SEROTONIN (5-HT)

The uptake of monoamines such as DA and 5-HT is similar in some respects for both brain synaptosomes and blood platelets. Accumulation of monoamines in both platelet (Solomon et al, 1970; Trenchard et al, 1975; Rudnick et al, 1980) and brain synaptosome (Hamberger and Tuck, 1973) is sensitive to tricyclic antidepressants such as imipramine, protryptyline and nortryptyline.

Tuomisto and Tuomisto (1979) studied the accumulation of DA in striatal synaptosomes. These researchers arrived at a figure of 1.4×10^{-7}M as a Km for the uptake of DA by the synaptosomes. Gordon and Olverman (1978) demonstrated that the uptake of DA by human platelets is slow and presented evidence for a low affinity (Km = 100 µM) uptake process. Also documented in the paper by Tuomisto and Tuomisto are the inhibitory effects of imipramine and reserpine on DA accumulation. Both imipramine and reserpine significantly inhibited the uptake of DA in the synaptosome preparation.

Sneddon (1973) demonstrated that the uptake of 5-HT by blood platelets was dependent upon the presence of a Na^+ gradient (out > in). Heinz (1967), in a review of transport through biological membranes, described the need for the presence of Na^+ and K^+ for the accumulation of monoamines by striatal brain slices.

Uptake of monoamines by platelets (Abrams and Solomon, 1969) and brain synaptosomes (Heinz, 1967) is reduced at low temperatures (<37°C). Metabolic inhibitors such as ouabain and iodoacetate decrease uptake by platelets (Solomon et al, 1970) and brain synaptosomes (Cooper and Roth, 1974).

With respect to uptake of 5-HT by platelet membrane vesicles, Rudnick et al (1977, 1980) demonstrated that an inwardly directed sodium ion gradient could drive the accumulation of 5-HT. This uptake was highly sensitive to a tricyclic antidepressant, imipramine, and was independent of the presence of ATP. Rudnick (1980) also showed that isolated dense granules from platelets were insensitive to sodium ion concentration but uptake of 5-HT was inhibited by reserpine, a storage

inhibitor in aminergic neurons (Cooper and Roth, 1974). This granular storage would appear to be partially dependent on the presence of ATP in the maintenance of transmembrane pH gradient (Rudnick et al, 1980).

Much evidence is present in the literature to support the contention that DA and 5-HT do not traverse the platelet membrane by identical processes. Trenchard et al (1975), using protryptyline and clomipramine as uptake inhibitors, demonstrated that DA and 5-HT uptake are not inhibited to the same degree. Work by Airaksinen et al (1980) revealed that replacement of side chain subgroups on various β-carbolines produced analogs, some of which affect the accumulation of DA and 5-HT to different extents.

The blood platelets from patients with certain neurological disorders such as Huntington's chorea, Down's syndrome, Parkinson's disease show abnormal uptake of some biogenic amines. Boullin and O'Brien (1970) and Barbeau et al (1975) reported that platelets from patients with Parkinson's disease showed abnormal DA uptake. The uptake of 5-HT by platelets from these patients appeared normal. McLean and Nihei (1977) reported an abnormally high accumulation of DA by platelets from patients with Huntington's chorea. Platelets from patients with Down's syndrome show decreased 5-HT uptake as reported by McCoy et al (1974). These researchers also observed a decreased ATPase activity and increased Na^+ content in platelets from these patients.

Work by Gordon and Olverman (1978) indicated that 5-HT is taken up very efficiently by an active transport process ($K_m \simeq 1$ μM). Further, DA accumulation is driven by a low affinity process ($K_m \simeq 100$ μM). DA was shown to inhibit 5-HT transport competitively

and vice versa. In each case the K_i value for the inhibitory amine was similar to its own K_m for transport, suggesting that DA uses the same uptake carrier as 5-HT.

Born et al (1972) demonstrated that the inhibitory potency of drugs as antagonists to 5-HT platelet membrane binding does not correlate with their effectiveness as uptake inhibitors in platelets. Gordon et al (1977) pointed out that this tends to cast doubt on the 'single receptor' hypothesis.

The existence of a transmembrane pH gradient (interior acidic) appears to play an important part in the transport of amines into blood platelets (Rudnick et al, 1980). Carty et al (1981) found that isolated dense granules exhibited an intragranular pH of approximately 5.4. When placed in a buffered medium at neutral pH, amine uptake was observed. Proton ionophores such as carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and NH_4Cl collapsed the transmembrane pH gradient resulting in efflux of stored amine to extracellular space. The transmembrane potential of the platelet granule proved to contribute to amine storage.

The importance of a transmembrane pH gradient in amine accumulation was borne out by Nichols and Deamer (1976) using liposomes. Catecholamines were concentrated against a gradient and efflux of stored amine was accomplished in the presence of 10 mM NH_4Cl. The chromaffin granule ghost also presents an ammonium ion sensitive uptake of DA (Ingebretsen and Flatmark, 1978). An ATPase activity is believed to exist in the granule membrane which appears to act as an inwardly directed proton pump producing a pH gradient (Carty et al, 1977;

Phillips and Allison, 1978). DA stored through this proton translocating ATPase was released upon addition of proton ionophores such as FCCP, NH_4Cl and carbonyl cyanide m-chlorophenylhydrazone (CCCP).

The blood platelet dense granule exhibits phenomena akin to chromaffin granule transport and storage. Johnson and Scarpa (1981) presented evidence for an electron transport chain in platelet dense granules which bears striking resemblance to the electron transport chain found in adrenal chromaffin granules. This electron transport chain may be associated with the accumulation of biogenic amines.

E. CATECHOLAMINE RECEPTORS

In 1948, Alquist set the stage for the present day identification of neuronal receptors. He did so by dividing adrenergic receptors into two classes (α and β) based on the rank order of potency of various agonists.

Originally, Alquist had proposed the following order of effectiveness of catecholamine analogues to identify α-adrenergic receptors:

L-adrenaline > L-noradrenaline > α-methyl-noradrenaline > α-methyl-adrenaline > isoprenaline

Today, this schema is more or less followed although the identification of receptor subtypes has made the task of classification difficult. The α and β receptors have now been broken down into α_1/α_2 and β_1/β_2 subtypes. These classifications are based upon the rank order of potency of various agonists and antagonists to block the receptor site.

Interconversion of the subtypes has shown susceptibility to changes in the chemical composition of the incubation medium. Guanine nucleotides seem to initiate conversion from the high affinity receptor subtype to the low affinity subtype (Smith and Limbird, 1981: Lynch and Steer, 1981). Lynch and Steer (1981) indicated using [^{3}H] - norepinephrine and [^{3}H] - phentolamine that the existence of two receptor subtypes was feasible based on agonist-specific inhibition of

binding. Hoffman et al (1980), using computer modeling techniques indicated that high and low affinity α-receptor subtypes were likely to exist.

The identification of the low affinity α-subtype proved difficult in the past due likely to the freeing of ligand from α-receptor during washing procedures. Tsai and Lefkowitz (1978) have presented evidence that physiological concentrations of monovalent cations can inhibit binding of ligand to high affinity sites. It is believed that interconversion of the high and low affinity α_2-receptor sites could produce such effects. These researchers also found that Ca^{++} had no effect on agonist specific inhibition of binding while certain concentrations of Mg^{++} enhanced agonist interference.

Scrutton and Wallis (1981) identify the receptor on its susceptibility to various analogues in relation to other receptor subtypes. That is to say, an α_1 receptor should be stimulated selectively by an α_1 agonist and competitively inhibited by an α_1 antagonist more readily than by an α_2 antagonist (Table 1). These effects should demonstrate sterospecificity.

Newman et al (1978) using whole cells and platelet homogenates calculated the K_D for DA binding to be 3.1 μM based on its inhibition of digydroergocryptine (DHEC) binding. Acceptance of such a calculated figure predisposes the assumption that both compounds are binding to the same class of site. Lynch and Steer (1981) studying α_2 receptor sites through the binding of epinephrine and phentolamine obtained a K_D for DA in excess of 19 μM based on its inhibition of ligand binding.

TABLE 1

Classification of adrenoreceptors. [Taken from Scrutton and Wallis, 1981]

TABLE 1

CLASSIFICATION OF ADRENORECEPTORS

Sub-type Selectivity	Agonists	Antagonists
$\alpha_1 = \alpha_2$	noradrenaline	phentolamine
	adrenaline	dihyroergocruptine
	naphazoline	dihydroergotamine
$\alpha_1 > \alpha_2$	methoxamine	phenoxybenzamine
	phenylephrine	clozapine
		indoramin
		azapetine
		prazosin
		corynanthine
		WB-4101
$\alpha_2 > \alpha_1$	clonidine	yohimbine
	UK-14304	rauwolscine
	σ-methyl-noradrenaline	
	oxymetazoline	
	guanabenz	
$\beta_1 = \beta_2$	isoprenaline	propranolol
		oxyprenolol
		pindolol
		alprerolol

TABLE 1 (cont.../)

CLASSIFICATION OF ADRENORECEPTORS

Sub-type Selectivity	Agonists	Antagonists
$\beta_1 > \beta_2$	tazolol	practolol
	prenalterol	atenolol
		metaprolol
		tolamolol
$\beta_2 > \beta_1$	salbutamol	salmefamol
	metaproterenol	
	terbutaline sulphate	
	clenbutarol	
	pirbutarol	

CHAPTER III

METHODOLOGY

A. COLLECTION OF BLOOD

Venous blood was collected from healthy volunteers who had not ingested any drug one week prior to venipuncture. The blood was collected in 30 ml polycarbonate tubes containing 3.0 ml of acid-citrate-dextrose (Trenchard et al, 1975). The blood was spun at 200 xg for 5 minutes and the platelet-rich-plasma (PRP) removed using a plastic pipette.

B. COLUMN SEPARATION

The column separation method was performed according to Tangen et al (1971). A 12.5 cm x 2.5 cm plastic column containing Sepharose 2B (Pharmacia Chemicals, Uppsalla, Sweden) was used to separate the plastelets from plasma. The column was equilibrated with either modified Tyrodes solution ($NaHCO_3$, 11.90 mM; $NaH_2PO_4 \cdot 7H_2O$, 0.36 mM; dextrose, 5.55 mM; EGTA, 0.1 mM; KCl, 2.68 mM; $MgCl_2$ $6H_2O$, 0.49 mM; NaCl, 136.89 mM; pH 7.4) for uptake studies or NaCl, 140 mM; Tris·HCl, 10 mM; pH 7.4 for binding studies.

PRP was layered onto the equilibrated column and the platelets were eluted with the buffer used to equilibrate the column. The speed of elution was adjusted to 1 drop per 10 seconds.

Absorbance (260 nm) was monitored using a Uvicord model 2138 monitor (LKB, Sweden) and the light transmission recorded with a

Brinkman model 2543 potentiometric recorder (Brinkman Instruments, Ontario, Canada).

Platelets were collected in polycarbonate tubes and the number of platelets determined per ml buffer using a Coulter Counter model ZBI (Coulter Electronics).

C. UPTAKE EXPERIMENTS

Aliquots (200 µl each) of platelets collected in modified Tyrodes buffer were dispensed in 1.5 ml polypropylene tubes. The platelet concentration was adjusted to 1.5 - 4.0 x 10^8 cells per ml buffer.

Tritium labelled ligand was added to the tubes at given concentrations along with various inhibitors. The concentration of NH_4Cl used was 10 mM unless otherwise stated. Haldol was used at concentrations up to 10 µM. The inhibitory effects of imipramine were observed up to 15 µM.

The assay mixture was incubated a 37° C for varying time periods; depending on the ligand under study. Time course experiments were conducted to determine the equilibrium point of uptake. This incubation period was used throughout the uptake experiments. Equilibrium was defined as the state at which there is no net movement of ligand into or out of the platelet. Such a state is assumed to be represented by the amine level in platelets following incubation for sufficient time with a given concentration of ligand. In practice, the intraplatelet levels of 5-HT and DA were observed to reach a plateau after 15 and 60 minutes, respectively.

D. ASSAY PROCEDURES

Double Isotope Method

Using [^{14}C] - inulin or [^{14}C] - sucrose as indicators of extracelllular fluid, the amount of ligand taken up was determined (Barbeau et al, 1975). After incubation for the appropriate time period, ice-cold [^{14}C]-inulin (5.12 mCi/mmol) in 0.9% NaCl was dispensed in 1.0 ml quantities in each tube to stop transport of ligand. The tubes were then centrifuged at 15,000 x g for 5 minutes and 4° C using an Eppendorf model 5412 microcentrifuge. A 10µl aliquot of the supernatant was removed to a scintillation vial to which was added 10 ml Aquasol II. The remainder of the supernatant was removed using a 25 gauge needle and syringe. The platelet pellet was dissolved overnight in 200 µl of 1% sodium dodecylsulphate (SDS) at room temperature. The suspension was removed to a scintillation vial and a further 200 µl of 1% SDS used for washing the tube was added to the vial.

Samples were counted using a Beckman model LS-230 liquid scintillation counting apparatus. Channel A discriminator was adjusted to ^{3}H range and channel B discriminator adjusted to ^{14}C range. The samples were counted for 10 minutes on two separate occasions and results presented as counts per minute (cpm).

The data was analyzed using a Hewlitt Packard model 34-C calculator using a program based on the following formula:

$$Bp - Bs \frac{Ap - aBp}{As - aBs} = \text{cpm of radioactivity labelled amine in pellet.}$$

Where Ap = cpm of precipitate in channel A, a is the ratio of the ^{14}C counts in channel A over channel B to account for spillover, A_S = cpm of supernatant in channel A, and Bp and Bs represent cpm of precipitate and supernatant, respectively in channel B. This formula calculates the amount of intracellular ligand by estimating the amount of extracellular fluid counts and subtracting it from the total counts. The specific activity of the ligand was used to determine the quantity of ligand taken up in nmols per unit sample volume.

This method proved to be extremely accurate. However, the inulin and sucrose were prone to bacterial degradation. This could, at times, produce erroneous results.

GLASS FILTER METHOD

A modified method of Newman et al (1977) was tested to see if it is suitable for assays of amine uptake in platelets as well as amine binding to platelet membranes. After incubation of the platelet suspension for the appropriate period of time, the tubes were removed to ice and 1.0 ml of icecold 0.9% saline added. The tubes were vortexed and the mixture filtered through whatmann GF/C or GF/A glass fiber filters. There was no appreciable difference in the results obtained between these two filters. The major difference was the extent of ligand binding to the filter itself. In all cases this background binding was calculated and taken into account in the final analysis.

The tube was rinsed once with ice-cold isotonic saline and the filter washed further with 154 ml of ice-cold NaCl.

The filters were allowed to dry at room temperature and placed in scintillation vials with 0.5 ml Protosol. The vials were heated to 60°C for 30 minutes in a shaking water bath. They were then allowed to cool after which 50 µl glacial acetic acid was added to bring the mixture to neutrality. Econoflour (10ml) was added to each vial and allowed to equilibrate for 1 hour. The vials were then counted for 10 minutes in the tritium range and the results converted to pmols per 10^8 cells.

CENTRIFUGE AND SWAB TECHNIQUE

The glass fiber filter method proved inaccurate for the 5-HT uptake experiments due to the extensive washing procedure. The following method for estimation of 5-HT incorporation was employed (Solomon et al, 1970): The incubation period for 5-HT uptake was limited to 15 minutes. After 15 minutes incubation, the uptake levelled off. Ice-cold isotonic saline (1.0 ml) was added to each tube tube. The tubes were placed in ice and the samples centrifuged at 15,000 x g for 5 minutes in a 4°C cold room. The supernatant was drawn off through a 25 gauge needle. The tubes were then swabbed dry. The suspension was then transferred to scintillation vials. The tubes were washed with 30 µl SDS (1%) and 10 ml Aquasol II added to each vial. The vials were counted for 10 minutes in the tritium range and results expressed as nmol/10^8 cells.

E. MAO EFFECT ON DA STORAGE

The degree of DA degradation was determined by employing thin layer chromatography (TLC) techniques. The time of incubation for DA uptake measurements did not exceed 80 minutes. Therefore, the platelets were incubated with tritium labelled DA for 80 minutes in this experiment. The cells were then lysed and a 5 µl sample spotted onto LK5 silica gel, (Terochem laboratories, Edmonton). The following solvent system was used to determine the purity of the freed ligand: n-butanol: water: glacial acetic acid (12:5:3). Spot migration was observed under ultravoilet light in reference to samples of hot and cold DA. The path which the eluted spot traveled was divided into 1 cm sections and scraped into scintillation vials to which were added 10 ml Aquasol II. The purity of the ligand was expressed as percent cpm eluted spot in relation to the total count of the track.

F. MEMBRANE ISOLATION

Platelets, suspended in Tyrodes buffer were frozen overnight at -20°C. The frozen platelets were then thawed at room temperature.

Several different methods were evaluated in the production of an isolated membrane fraction. The membrane yield was estimated by measuring the amount of protein recovered in the membrane fraction from 3.0×10^9 platelets. The yields ranged from 60 µg/ml to 310 µg/ml. The nitrogen bomb method produced the highest yield.

POLYTRON METHOD

This method is a modification of the method presented by Bruns et al (1980) and is as follows: After thawing the platelet suspension, the cells were centrifuged down at 5,000 x g for 10 minutes and resuspended in Tris buffer. The cells were then subjected to maximum power Polytron treatment for 30 seconds. The resulting suspension was centrifuged at 2,000 x g to remove whole cells, the supernatant saved and the precipitate subjected to further polytron treatment. The pooled supernatants were then centrifuged at 9,000 x g in a Beckman model L3-50 ultracentrifuge using a 65 rotor for 15 minutes. This step removed whole cells and subcellular organelles. The supernatant was then centrifuged at 40,000 x g for 30 minutes to collect the membrane fraction.

GLYCEROL SHOCK TREATMENT

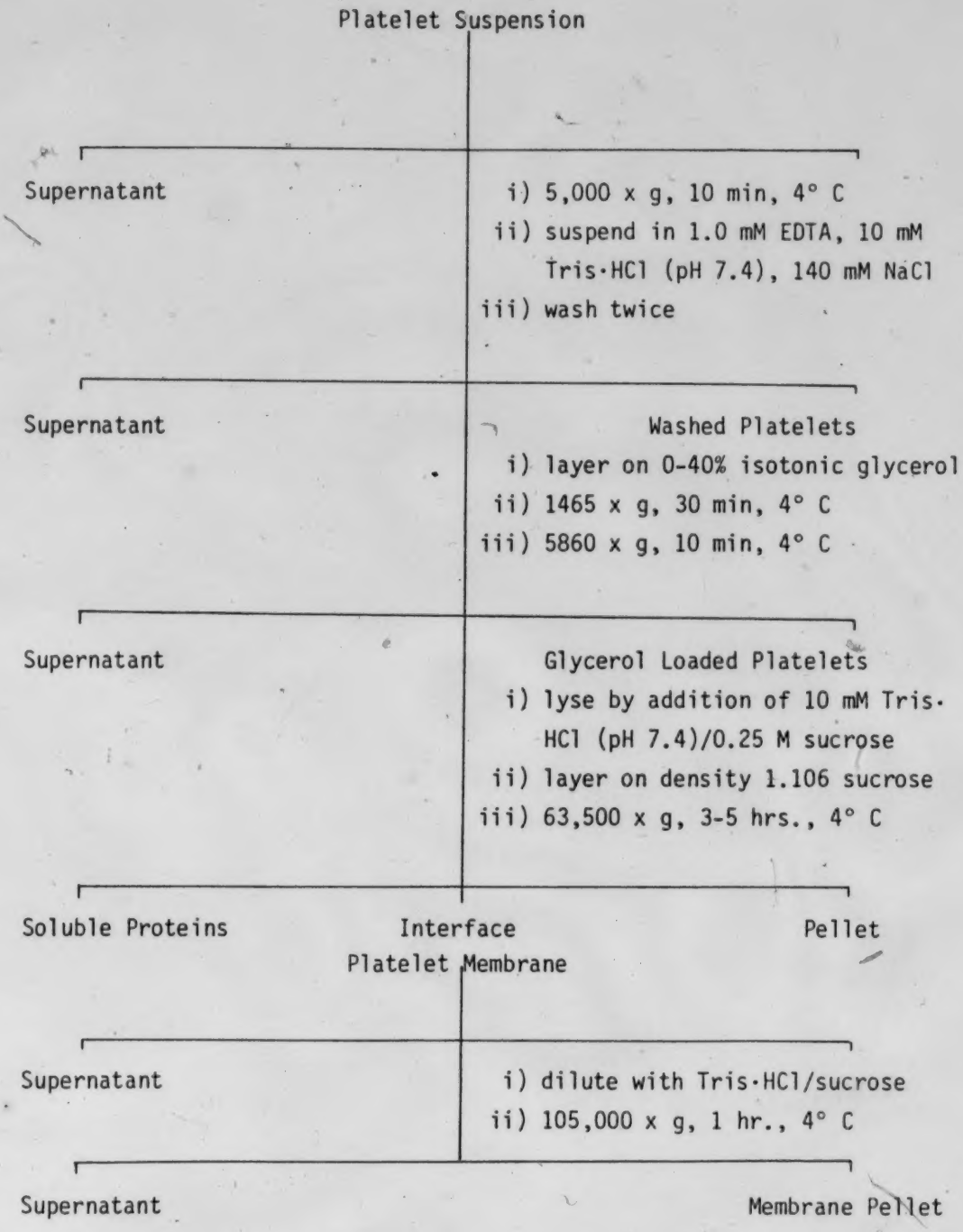
Using the method of Barber and Jamieson (1970) the platelet suspension was subjected to the regimen outlined in figure 1. Although this method resulted in very pure preparations of membrane, the amount of membrane recovered was low.

NITROGEN BOMB TREATMENT

This method of membrane isolation was originally described by Weglick et al (1980) involving the isolation of cardiac sarcolemma. Column washed platelets, frozen overnight at -20°C were thawed at room

Figure 1

Regimen for membrane isolation by the glycerol shock treatment of Barber and Jamieson (1970)


Platelet Suspension
Supernatant
i) 5,000 x g, 10 min, 4° C
ii) suspend in 1.0 mM EDTA, 10 mM Tris·HCl (pH 7.4), 140 mM NaCl
iii) wash twice
Supernatant
Washed Platelets
i) layer on 0-40% isotonic glycerol
ii) 1465 x g, 30 min, 4° C
iii) 5860 x g, 10 min, 4° C
Supernatant
Glycerol Loaded Platelets
i) lyse by addition of 10 mM Tris·HCl (pH 7.4)/0.25 M sucrose
ii) layer on density 1.106 sucrose
iii) 63,500 x g, 3-5 hrs., 4° C
Soluble Proteins
Interface
Platelet Membrane
Pellet
Supernatant
i) dilute with Tris·HCl/sucrose
ii) 105,000 x g, 1 hr., 4° C
Supernatant
Membrane Pellet

temperature. The resulting platelet suspension was subjected to 2,000 pounds per square inch (psi) nitrogen gas in a nitrogen bomb apparatus (Parr Instrument Company, Illinois). Allowing 20 minutes for maximum nitrogen penetration, the suspension was released into 10 mM tetra - sodium pyrophosphate (pH 7.0) at room temperature. This suspension was then centrifuged at 40,000 x g for 30 minutes to pellet the membrane portion of the lysate. This lysate was washed 3 times with 75 mM Tris buffer. The membrane suspension was frozen at -20°C overnight for use the next day.

PERCOLL GRADIENT

Platelet membranes were isolated using the method of Gogstad (1980). After production of the platelet lysate by nitrogen bomb method, the heterogeneous mixture was layered onto a Percoll step gradient (67.5%/ 73.0%) and spun at 6,000 x g for 20 minutes. The membrane containing layer was collected at the interface and suspended in 67.5% Percoll and spun at 79,000 x g for 20 minutes resulting in a self generating continuous density gradient.

Fractions (100 µ l) were collected and subjected to binding experiments.

The time required to remove the Percoll from the membrane layers proved extremely costly; therefore this method was abandoned. Platelets (5×10^7 cells) were incubated with 0.5% Triton X-100 for 1 hour at room temperature. The resulting suspension was layered onto a 1.2 x 14 cm DEAE Sephacryl 300 column. The suspension was eluted with

140 mM NaCL; 10 mM Tris HCl (pH 7.4); 1 mM EDTA; and the flow rate adjusted to 1 drop/10 seconds. Fractions of 100 µl were collected and the absorbance (260 nm) monitored.

G. PROTEIN DETERMINATION

Protein was determined using amido black as an indicator. To 100 µl of sample 1% SDS was added to a final concentration of 0.1% and incubated for 10 minutes. Trichloroacetic acid (TCA) was then added to a final concentration of 15% for more than two minutes. The sample was filtered through Millipore HAWP 0.45 µm filters and washed with 3 ml of 6% TCA. The filters were removed to a beaker containing 0.1% amido black in methanol: glacial acetic acid: water (90:2:8 volume %). Destained filters were washed 1-2 minutes in distilled water then blotted dry on filter paper. The protein-dye complex was then extracted from the filters with 2-3 mls of 25 mM NaOH, 0.05 mM ethylenediaminetetraacetic acid (EDTA) in 50% aqueous ethanol until all color was removed. Optical density was determined at 630 nm. Bovine serum albumin was used as the protein standard.

H. BINDING ASSAY

Binding assays were performed according to Newmann et al (1977). Membrane protein concentrations were adjusted to approximately 300 µg/ml, using 75 mM Tris·HCL (pH 7.4). Sample volumes of 100 µl were dispensed into 1.5 ml polyproplylene tubes to which was added

varying concentrations of ligand. After an appropriate incubation period the samples were diluted with ice-cold isotonic saline and filtered through Whatmann GF/C filters. The membrancces were washed with 15 ml of ice-cold 0.9% NaCl and allowed to air dry. The filters were then placed in scintillationvials containing 0.5 ml Protosol and heated to 60°C for 30 min in a shaking water bath. After cooling 50 µl of glacial accetic acid was added to neutralize the solution. The vials were then filled with 10 ml Econoflour and allowed to equilibrate for 1 hour. Counts were expressed as femtomoles (fmols) bound per milligram protein. Specific binding was defined as that which could be displaced by greater than 100 x the original ligand concentration.

Binding data were analyzed following the method of Scatchard (1949) and Hill (1913).

I. MATERIALS

[^{3}H]-DA (40 Ci/mmol) and [^{3}H]-5-HT (16.6 Ci/mmol) were purchased from Amersham Radiochemical Centre. Haloperidol was obtained from McNeil Laboratories (Canada). Imipramine was purchased from Sigma Chemicals. All other materials were of the highest purity commercially available.

CHAPTER IV

RESULTS

A. UPTAKE EXPERIMENTS

Washed platelets were chosen for the uptake experiments to provide a uniform environment in which to observe the incorporation of ligand. This approach, we felt, would minimize variation among volunteers revealed by earlier researchers.

The choice of buffers utilized to elute the platelets is extremely important. Platelets eluted with NaCl/Tris buffer show abnormally high uptake of DA. This could possibly be caused by an imbalance in the ion gradients across the membrane. To test this possibility potassium was added to the incubation medium in increasing concentrations and its effect on accumulation monitored. Figure 2 shows that increasing concentrations of potassium ion, up to levels found in the plasma, decrease incorporation. We see, also, that ammonium chloride exerts a uniform effect over the concentration range described. The ammonium chloride effect will be considered in further detail in subsequent sections. Modified Tyrodes buffer was used throughout the uptake experiments since it produced uptake values similar to those obtained by platelets in plasma.

Figure 3 shows the elution pattern of platelets. A moderate separation was achieved when 15 ml of PRP was layered onto the Sepharose column. The platelet sample was collected between the two arrows on the graph. This was done to minimize mixing of cells and plasma proteins.

Figure 2

Potassium ion effect on DA uptake. Increasing concentrations of K^+ were added in the presence and absence of NH_4Cl (10mM) and the amount of DA accumulation determined. [DA] = 22.7 µM. Uptake conditions as outlined in procedure section.

(●) uptake without NH_4Cl; (▲) uptake with NH_4Cl

(■) uptake with NH_4Cl subtracted from uptake without NH_4Cl

Points represent the mean of triplicate samples from one experiment.

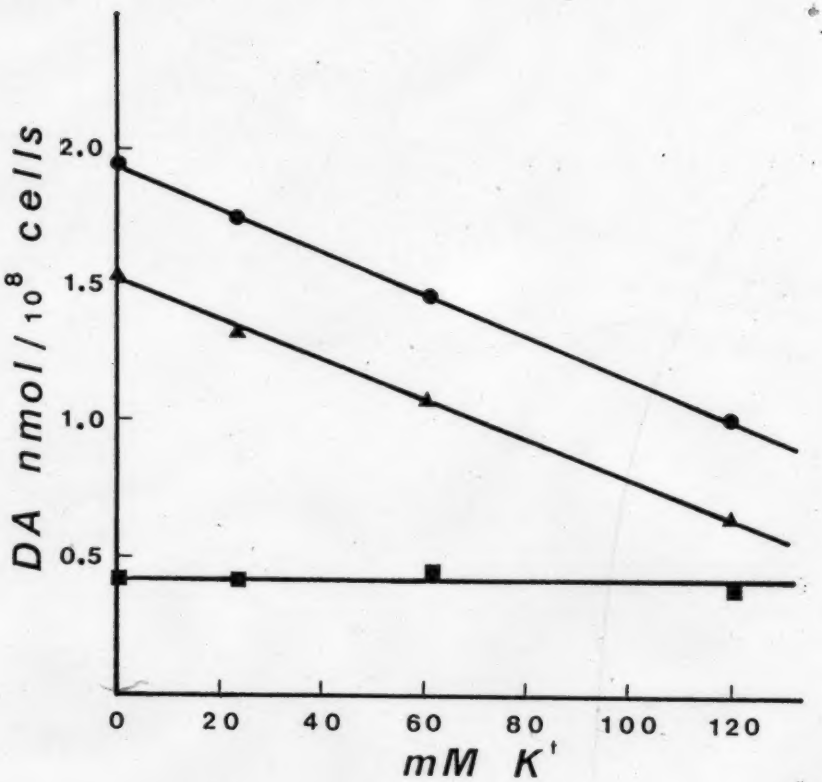
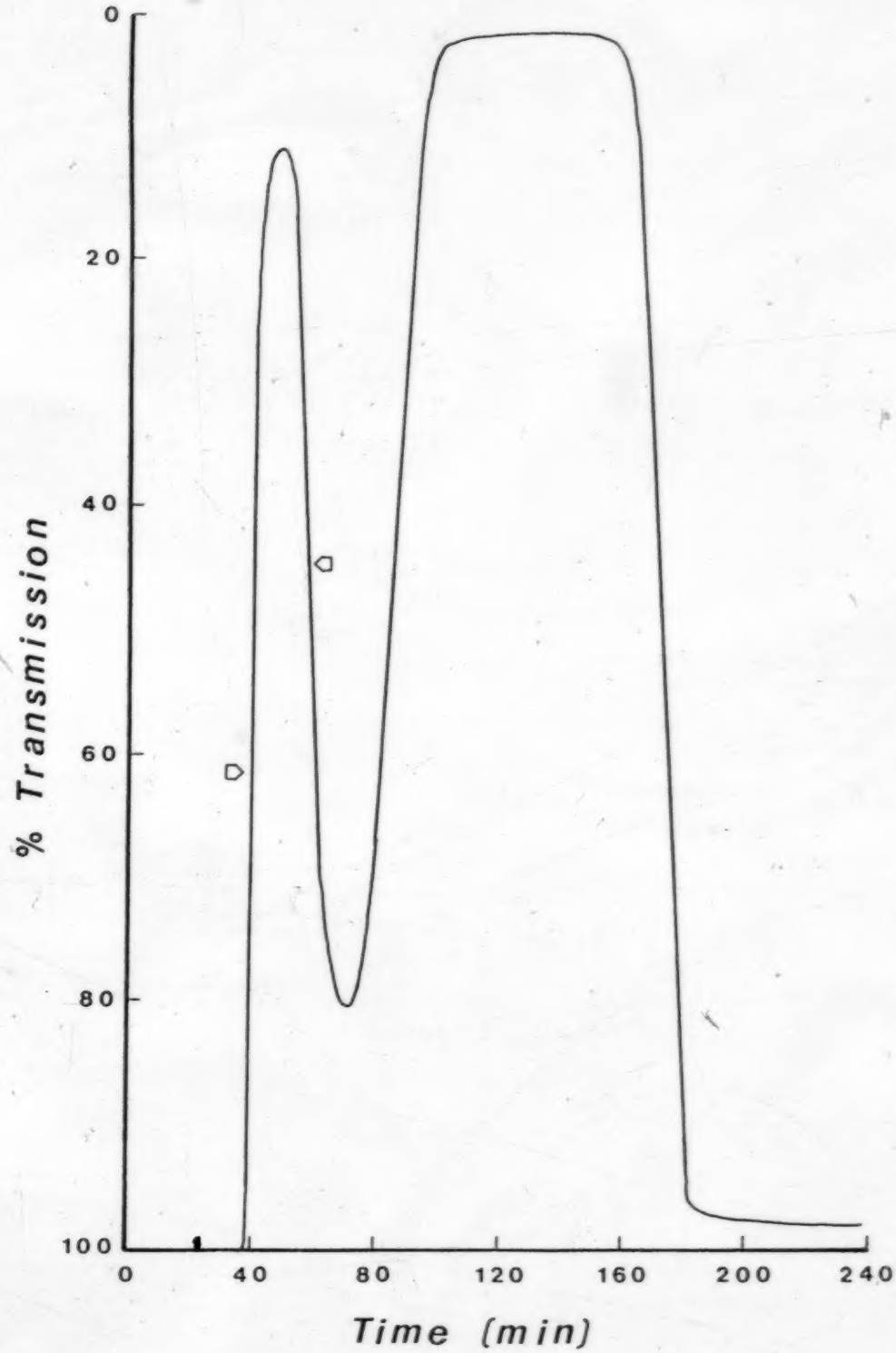

DA nmol/10^8 cells
2.0
1.5
1.0
0.5
0
20
40
60
80
100
120
mM K+

Figure 3

Separation of platelets from plasma. Cells were separated on a Sepharose 2B column as outlined in the Methodology section. The arrows indicate the region in which the cells were collected. Transmission was monitored at 260 nm.


0
20
40
60
80
100
% Transmission
0
40
80
120
160
200
240
Time (min)

The speed of elution was important for several reasons. Firstly, if the travel time in the column is too long, platelets are likely to degranulate thus changing their internal makeup and influencing other cells in the vincinity. If the speed of elution is too rapid, inadequate separation of platelets and plasma proteins is realized. Platelets often undergo shape change and extrusion of granular contents with rapid passage through the column.

The time of elution demonstrated on the tracing proved to be the most acceptable in that platelet activation was minimized. Samples examined under phase contrast microscopy indicated little platelet excitation.

The degree of platelet excitation can also be demonstrated by preloading the platelets in plasma with tritium labelled 5-HT. The cpm/10^8 cells, post elution, is then compared to cells loaded for the same period of time in plasma. This method evidenced the same qualitative results as the microscopic technique but proved time consuming and costly.

The degree of activation of the platelet was monitored using phase contrast and flourescence microscopy. Skaer et al (1981) reported the use of mepacrine, an antimalarial drug, to specifically label dense granules of platelets. These granules are easily observed under flourescence microscopy. Mepacrine (1 μ M) allows one to determine the extent of activation by observing the relative number of granules per field before and after passage through the Sepharose column.

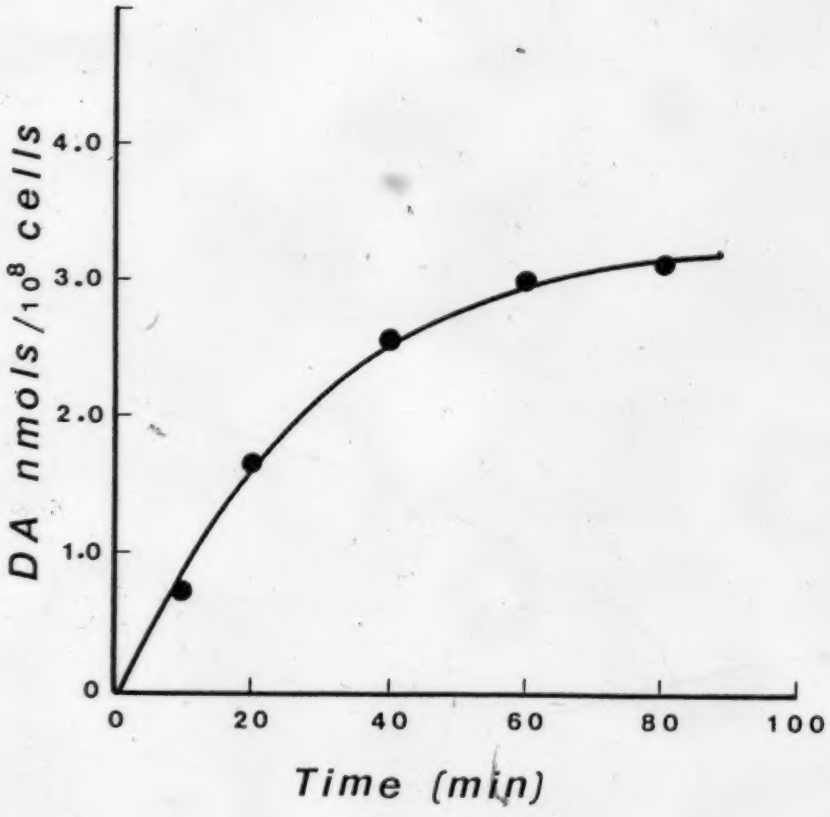
A time course for the uptake of DA was obtained to determine the incubation period required to reach a state of equilibrium. As indicated in the materials and methods section the equilibrium state was defined as that at which there was no net movement of ligand into or out of the cell. Figure 4 illustrates the time needed for uptake to reach equilibrium at a fixed concentration of ligand.

As demonstrated in figure 4, the equilibration time for DA is approximately 60 minutes at 37°C. This is in good agreement with times determined by previous researchers (Solomon et al, 1970; Boullin and O'Brien, 1970). 5-HT is rapidly taken up having an equilibration time of approximately 15 minutes (Boullin and O'Brien, 1970, Gaetano, 1977).

Due to the relatively high concentration of MAO in the human platelet, the possibility exists that DA is broken down over a long incubation period. The extent of DA breakdown was assessed using thin layer chromatography. Platelets incubated with tritium labelled DA for 1 hour were lysed using the Polytron. The debris was sedimented at 40,000 x g for 20 minutes. Supernatant from the 40,000 x g spin was used to determine the extent of metabolic breakdown of this catecholamine. After 60 minutes incubation, greater than 95% of the total radioactivity of the spot track was located in the spot identified under ultraviolet light. The Rf value of the spot corresponded to that of labelled and unlabelled DA.

Figure 4

Time course of DA uptake. Column washed platelets were incubated at 37°C with tritium labelled DA. At the indicated time intervals 200 µL aliquots of the cell suspension were removed to 1 ml of ice-cold NaCl and assayed for stored ligand as outlined in the Methodology section. Each point represents the mean of triplicate samples from one representative experiment. The initial concentration of DA was 0.10 mM.


DA nmols/10^8 cells
4.0
3.0
2.0
1.0
0
0
20
40
60
80
100
Time (min)

The accumulation of the amines over a given concentration range was then examined. In an attempt to determine whether or not DA and 5-HT were incorporated via a pH gradient and/or monovalent cation dependent gradient, the effects of NH_4Cl were tested (figures 5 and 6). Addition of 10 mM NH_4Cl prior to the addition of labelled DA resulted in a linear DA concentration dependence of uptake ($r = 0.97$). Higher concentrations of NH_4Cl did not alter the uptake curve. Addition of ammonium chloride post incubation released accumulated amine. The internal level of amine dropped to that observed when NH_4Cl was added pre-incubation. Thus, it would appear that an ion gradient is necessary to maintain accumulated amine.

Uptake of 5-HT, on the other hand, evidences a component which is insensitive to ammonium chloride. As depicted in figure 6 ammonium chloride produced essentially no effect up to approximately 20 μM 5-HT, above which the uptake was inhibited linearly with concentration. It is possible that the 5-HT uptake observed at the lower concentrations could be produced by binding of 5-HT to the cell. In an attempt to clarify the matter, the effect of NH_4Cl on 5-HT binding was determined (data not shown). Ammonium chloride had no effect on ligand binding.

Since the accumulation of both amines was affected by the presence of ammonium chloride, transport inhibitors were employed to see whether DA and 5-HT traversed the plasma membrane by way of the same carrier.

Figure 5

Ammonium chloride effect on DA uptake. Platelets were incubated 1 hour at 37°C with radiolabelled DA and assayed as to the extent of DA uptake. NH_4Cl (10mM) was added either during or post (2 minutes) incubation and the amount of incorporated DA assessed. (●) uptake without NH_4Cl; (○) uptake with NH_4Cl. Each point represents the mean of three experiments with triplicate samples.

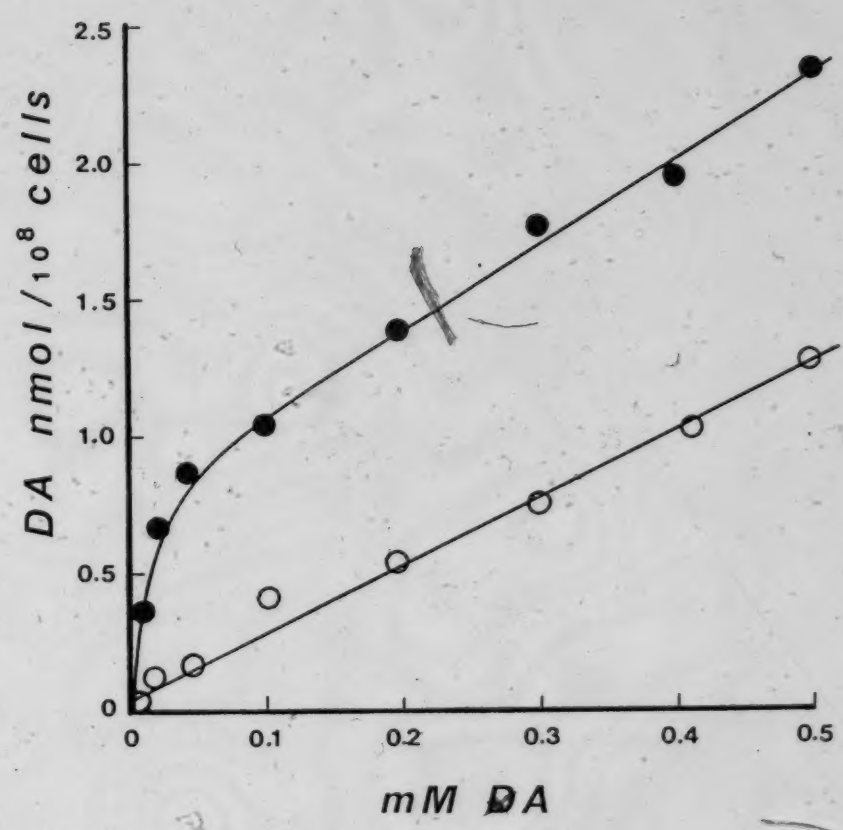
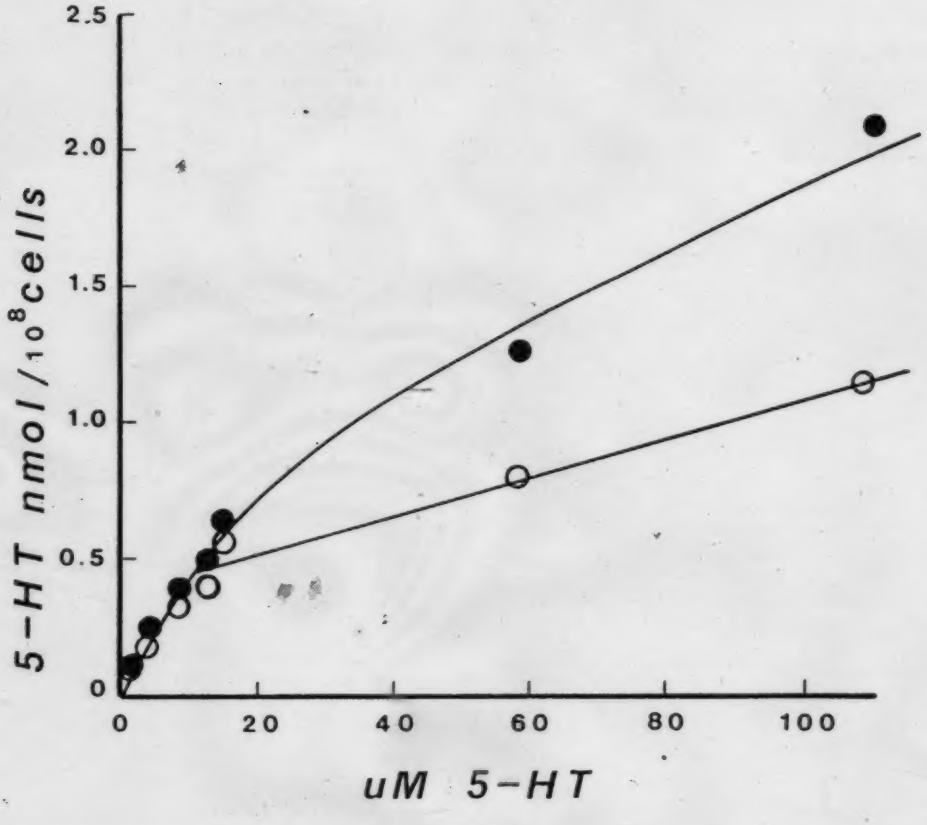

DA nmol/10^8 cells
2.5
2.0
1.5
1.0
0.5
0
0
0.1
0.2
0.3
0.4
0.5
mM DA

Figure 6

Effect of NH_4Cl on 5-HT uptake. NH_4Cl (10mM) was added either during or post (2 min) incubation to 100 µl aliquots of cell suspension and tritiated 5-HT at 37°C. The amount of incorporated 5-HT was calculated and expressed as nmols/10^8 cells.

(●) uptake without NH_4Cl; (○) uptake with NH_4Cl

Each point represents the mean of four experiments with triplicate samples.


2.5
2.0
1.5
1.0
0.5
0
5-HT nmol/10^8cells
0
20
40
60
80
100
uM 5-HT

Imipramine has been shown to be a specific transport inhibitor for 5-HT (Rudnick et al, 1980). Figure 7 demonstrates the effect of 1.5 μM imipramine on the uptake of 5-HT over the given concentration range. Under the influence of imipramine, the 5-HT uptake level increased linearly. When NH_4Cl (10 mM) was added to the incubation mixture in the presence of imipramine, there was no further release of accumulated ligand.

Imipramine (1.5 μM) was then tested as to its influence on DA accumulation. Figure 8 depicts dopamine uptake in the presence and absence of imipramine. Although affected to some degree, the presence of imipramine proved to be a small impediment to ligand uptake. Addition of ammonium chloride produced inhibition of accumulation in the presence of imipramine (data not shown). Thus, although DA is influenced to some extent by the addition of this tricyclic antidepressant, it does not evidence the great sensitivity of 5-HT to imipramine.

Creese et al (1981) described haldol as a specific DA antagonist in the brain. Kinetic studies of DA uptake in the past have shown haldol to have a pronounced effect (Solomon et al, 1970). Figure 9 shows the effect of 10 μM haldol on the accumulation of DA. Greater than 90% of uptake is inhibited over the DA concentration range tested. Increasing the concentration of haldol showed little change in its effect. As figure 10 illustrates, the consequence of added haldol (10 μM) on 5-HT accumulation was not as pronounced as on DA accumulation.

Figure 7

Effect of imipramine on 5-HT uptake. Uptake of 5-HT was determined in the presence (○) and absence (●) of 1.5 μM imipramine. Each point represents the mean of triplicate samples from one representative experiment. Each point represents the uptake at equilibrium as defined previously.

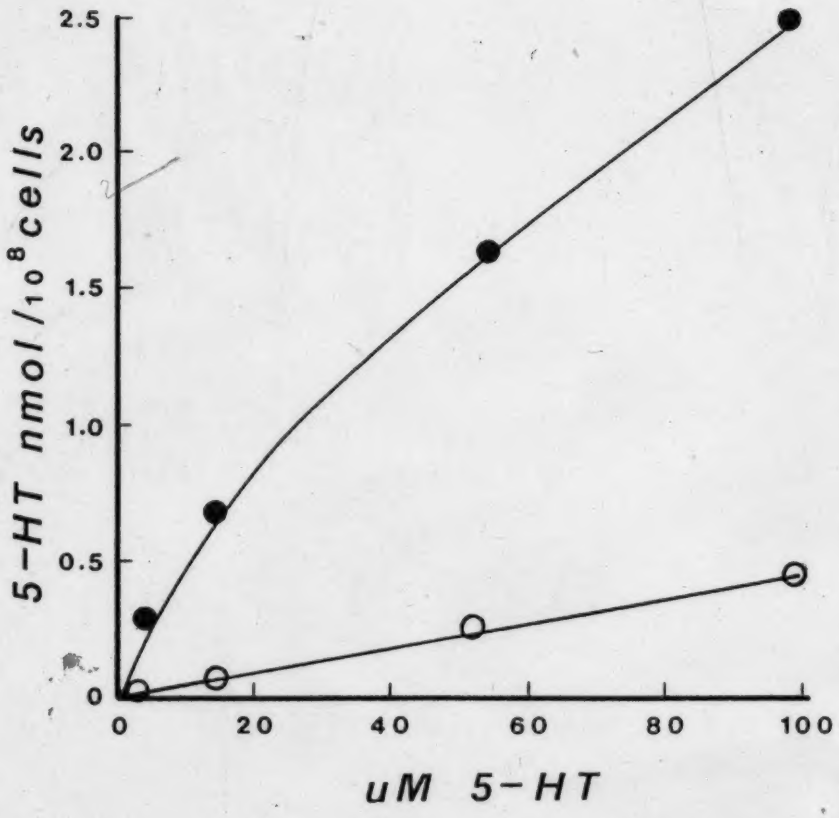

2.5
2.0
1.5
1.0
0.5
0
5-HT nmol/10^8 cells
0
20
40
60
80
100
uM 5-HT

Figure 8

Effect of imipramine on DA uptake. Uptake of DA was determined in the presence (○) and absence (●) of 1.5 μM imipramine. Each point represents the mean of triplicate samples from one representative experiment. Each point represents the uptake at equilibrium as defined previously.

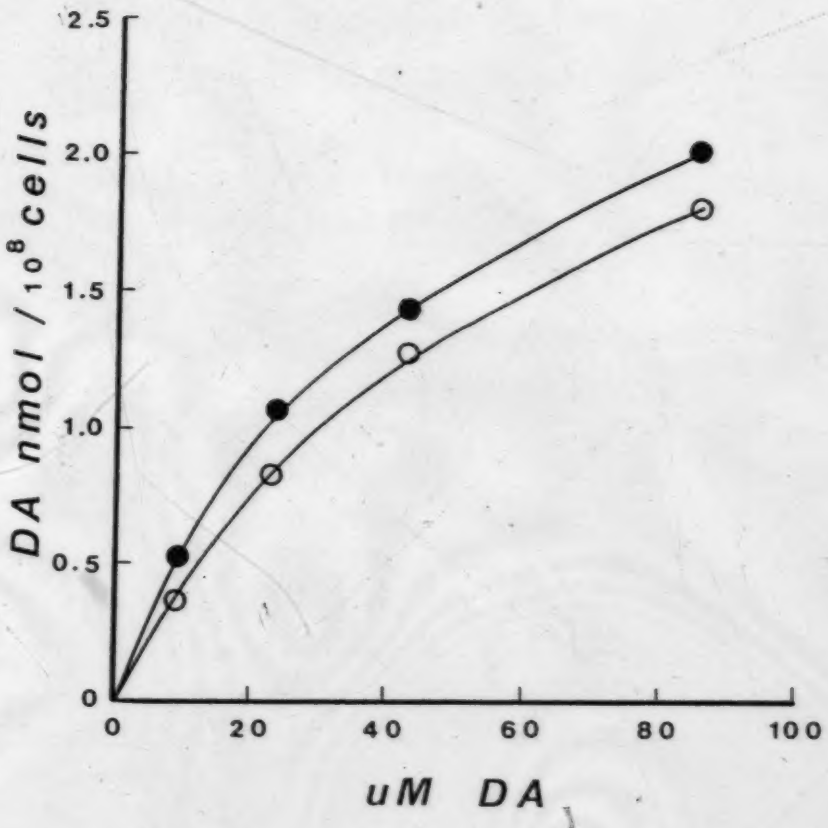

2.5
2.0
1.5
1.0
0.5
0
DA nmol / 10^8 cells
0
20
40
60
80
100
uM DA

Figure 9

Effect of haldol on DA uptake. DA uptake was quantitated over the concentration range given in the presence (○) and absence (●) of 10 μM haldol. Each point represents the mean of triplicate samples from one representative experiment.

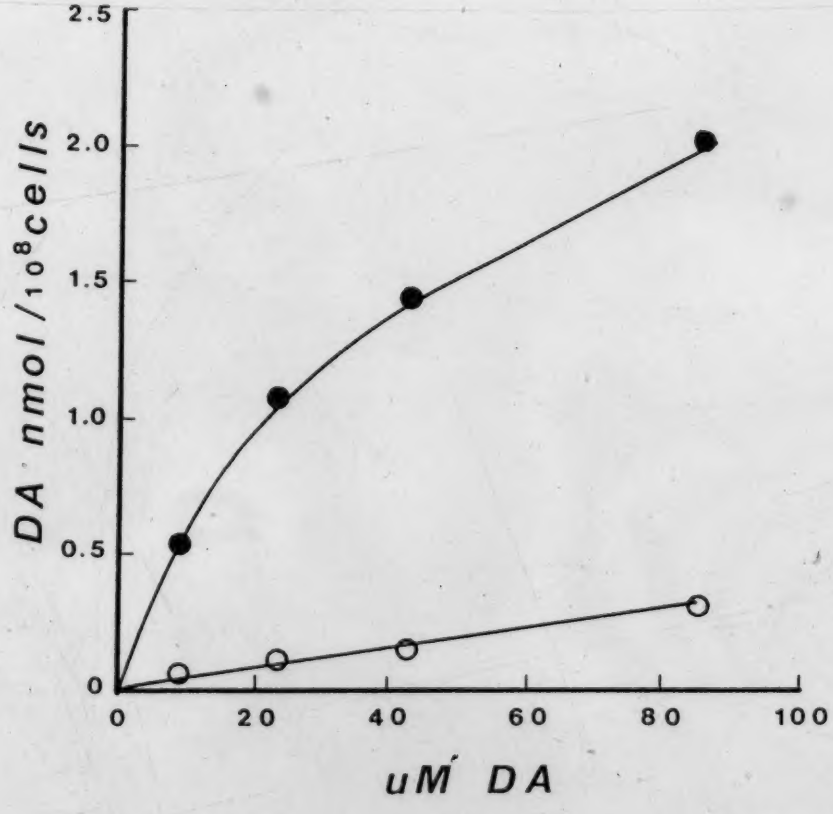
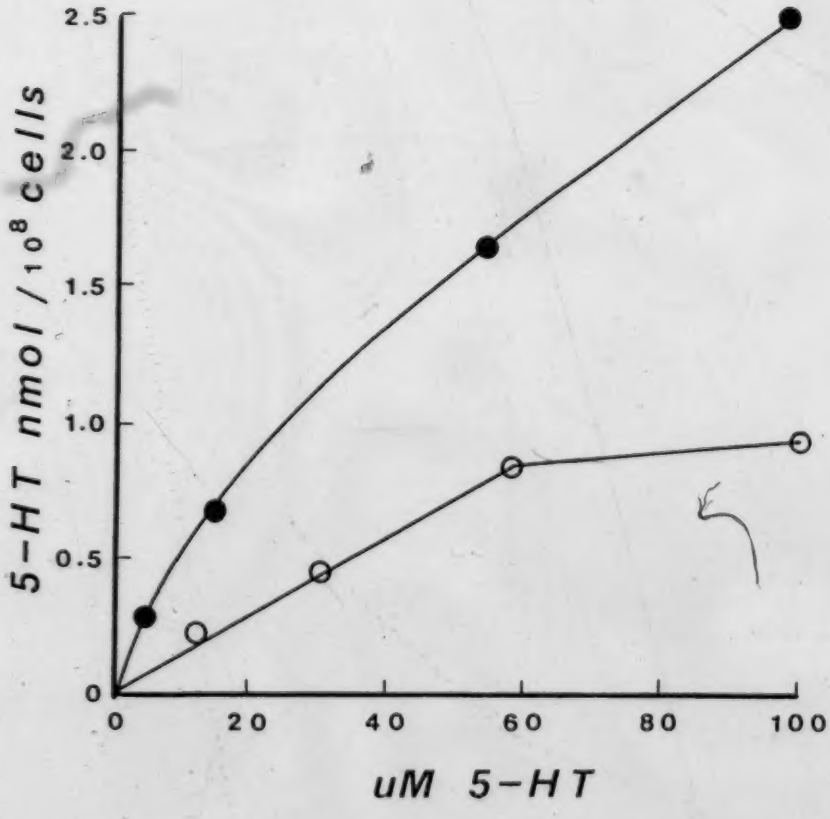

2.5
2.0
1.5
1.0
0.5
0
DA nmol/10^8cells
0
20
40
60
80
100
uM DA

Figure 10

Effect of haldol on 5-HT uptake. 5-HT uptake was determined over the concentration range in the presence (○) and absence (●) of 10 μM haldol. Points represent the mean of triplicte samples from one representative experiment.


2.5
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
5-HT nmol / 10^8 cells
uM 5-HT

To clarify the influence haldol has on the uptake of DA and 5-HT a study was undertaken comparing the inhibitory effect of varying concentrations of haldol on the accumulation at a fixed amount of amine. Figure 11 shows DA and 5-HT taken-up in the presence of various haldol concentrations. From this graph it is clear that haldol is a far better inhibitor of DA uptake, demonstrating an apparent K_i of approximately 180 nM. It appears that a 200 fold higher concentration of haldol is needed to inhibit 5-HT to the same extent. A similar plot of the effect of imipramine on DA and 5-HT uptake (figure 12) reveals results which do not conform to a simple competative inhibition model. This may be due to the multiple low affinity binding sites known to exist for 5-HT. However, it is clear that imipramine is a much better inhibitor of 5-HT uptake than DA uptake.

B. BINDING STUDIES

The platelet lysate obtained using the nitrogen bomb or polytron methods was fractionated using a DEAE Sephacryl column (14 x 1.2 cm). The different fractions obtained were tested for their binding ability to dihydroergocryptine (DHEC), an α-adrenergic agonist. Figure 13 shows a pattern of lysate elution from the column. The collected fractions were then subjected to binding analysis and expressed as cpm/mg protein (Figure 14). As seen in this figure, the highest binding per unit protein was seen with the fractions that were eluted in the latter half of the procedure.

Figure 11

Percent amine taken-up versus the log haldol concentration. Uptake of DA (●) and 5-HT (o) were determined in the presence of varying concentrations of haldol and expressed as a percentage of uptake in the absence of haldol. Points represent the mean of triplicate samples.

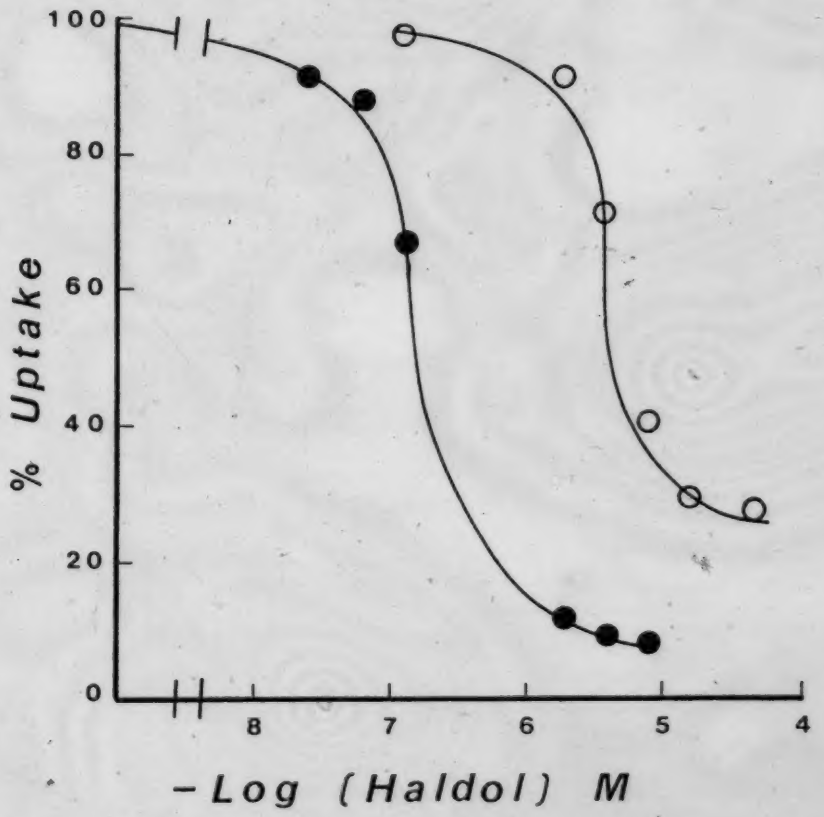

100
80
60
40
20
0
% Uptake
8
7
6
5
4
-Log (Haldol) M

Figure 12

Percent amine acccumulated versus the log imipramine concentration. Uptake of DA (○) and 5-HT (●) was determined in the presence of varying concentrations of imipramine and expressed as a percentage of uptake in the absence of exogenous imipramine. Points represent the mean of duplicate samples.

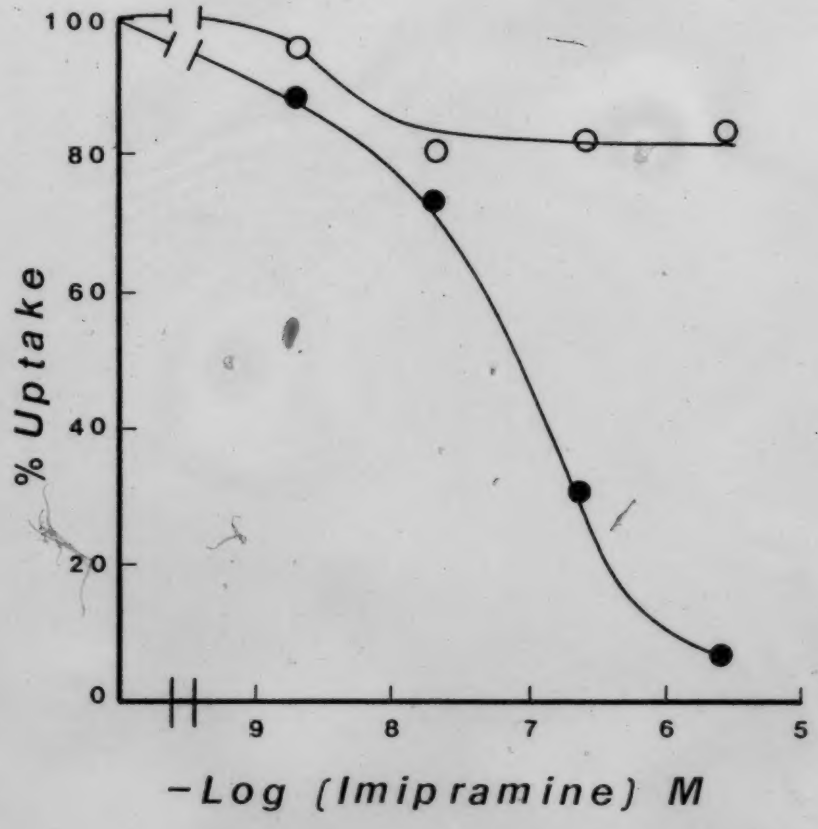

100
80
60
40
20
0
% Uptake
9
8
7
6
5
-Log (Imipramine) M

Figure 13

Fractionation of platelet lysate. Platelet lysate (1 X 10^8 cells) was layered onto a DEAE Sephacryl column (1.2 cm X 14 cm) and eluted with 140 mM NaCl; 10 mM Tris-HCl (pH 7.4); 1 mM EDTA buffer. The absorbance (260 nM) was monitored and a trace of % absorbance versus time produced. Flow rate was 1 drop/10 sec. Full scale deflection was equivalent to 2 optical density units.

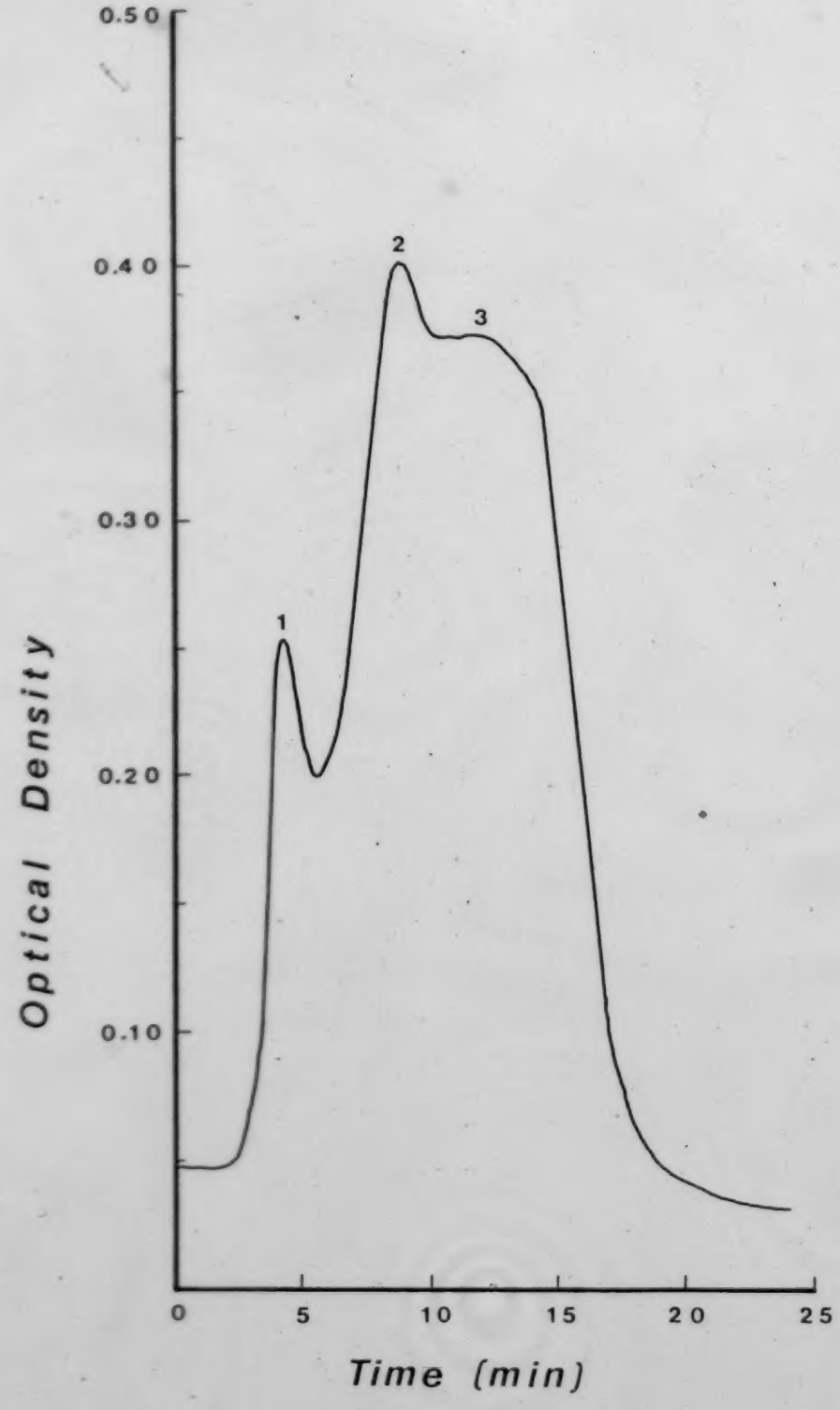
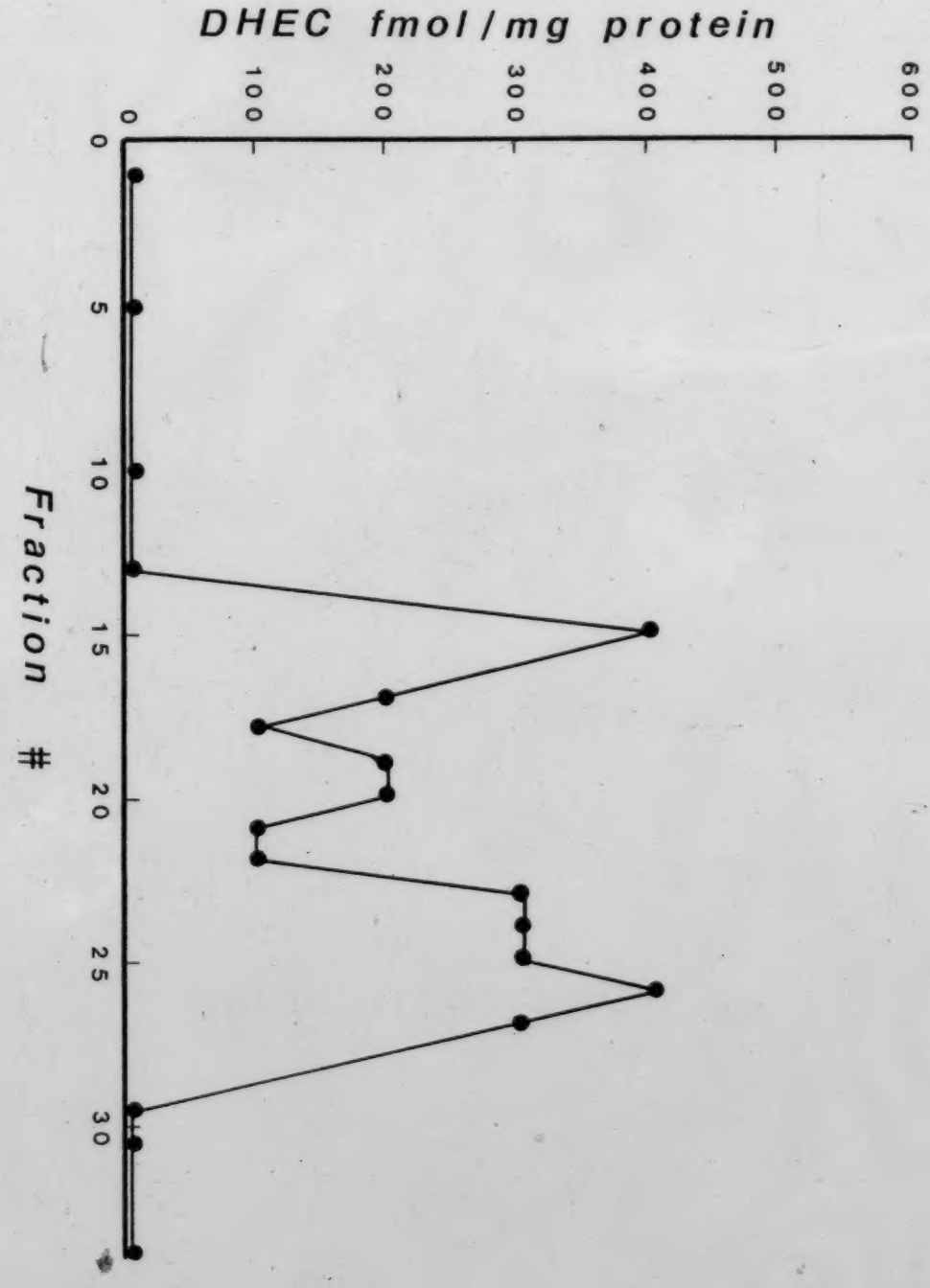

0.50
0.40
0.30
0.20
0.10
Optical Density
1
2
3
0
5
10
15
20
25
Time (min)

Figure 14

Binding of DHEC to fractionated lysate. Fractions (100 µl) collected from lysate indicated in figure 13 were subjected to DHEC binding (5nM). Results were expressed as cpm/mg protein.


DHEC fmol/mg protein
0
100
200
300
400
500
600
Fraction #
0
5
10
15
20
25
30

Although this method provides reliable results, the task of determining constants would be extremely cumbersome. A large amount of starting material is needed, not to mention the tremendous consumption of time. A centrifugation technique was, therefore, used to isolate the membrane fraction for binding studies.

A representative diagram of the membrane isolation procedure is presented in figure 15. Gogstad (1980) indicated the density of platlet membranes to be in the range of 1.095-1.100. Density marker beads (Pharmacia, Sweden) for Percoll gradients were utilized to determine the relative position of the membrane band. The gradient was then fractionated to isolate the membranes.

Binding studies using tritiated DHEC were attempted on this band. It was difficult to completely remove the Percoll beads from the isolated membrane layer. The presence of Percoll interfered with the determination of protein by the amido black method. To remove all traces of Percoll, the membrane layer had to be centrifuged at 100,000 x g for 1 hour.

In an attempt to characterize the membrane binding of DA, a crude membrane preparation was obtained by a differential centrifugation technique. The precipitate obtained from the 40,000 x g spin was used as a crude membrane preparation. The use of the nitrogen bomb procedure proved the most efficient. Figure 16 shows the time course of binding at 45 nM DA. The binding was conducted at room temperature.

Figure 15

Protocol for membrance isolation in a Percoll gradient. 3×10^9 platelets were lysed via the nitrogen bomb method and layered onto a Percoll step gradient (67.5%/73.0%) and spun at 79,000 x g for 20 minutes. The membrane containing layer was collected from the interface and layered for the second time onto a 67.5% solution of Percoll. This self generating continuous density gradient produced bands of varying density when spun at 100,000 x g for 15 minutes. Density marker beads were utilized to identify the membrane band. Percoll was removed from membranes by spinning at 100,000 x g for 60 minutes. Membranes were removed from the Percoll button formed at the bottom of the tube by gentle aspiration.

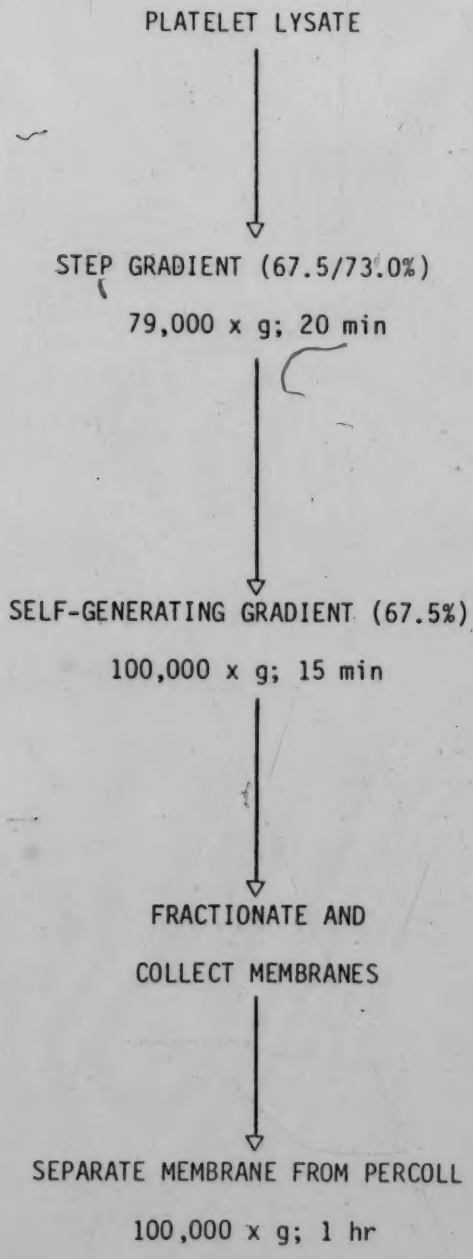
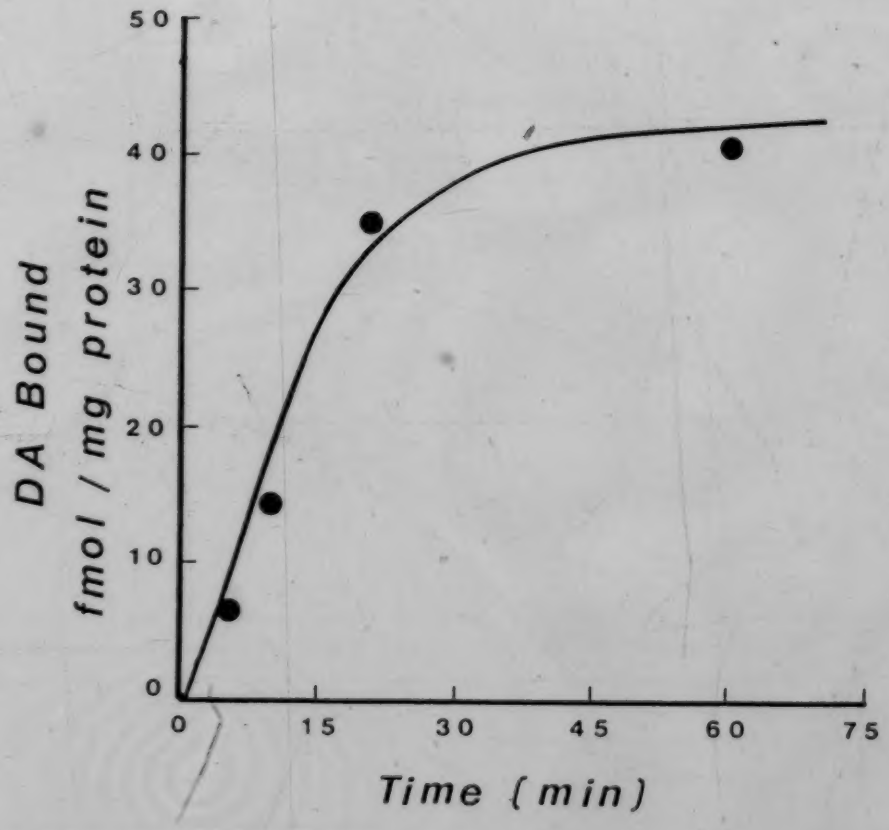

PLATELET LYSATE
STEP GRADIENT (67.5/73.0%)
79,000 x g; 20 min
SELF-GENERATING GRADIENT (67.5%)
100,000 x g; 15 min
FRACTIONATE AND
COLLECT MEMBRANES
SEPARATE MEMBRANE FROM PERCOLL
100,000 x g; 1 hr

Figure 16

Time course for DA binding to membranes. Membranes isolated using the nitrogen bomb/differential centrifugation techniques in 100 µl aliquots (300 mg/ml protein) were incubated with 45 nM DA over the time period indicated. The graph illustrates specifically bound ligand which was calculated as the amount of bound radiolabelled DA displaced by 1 µM unlabelled DA.


DA Bound
fmol / mg protein
50
40
30
20
10
0
0
15
30
45
60
75
Time (min)

As shown in the figure, the binding reaction appeared to be slow, reaching saturation levels in approximately 40 minutes.

Using this incubation time to achieve saturation, a binding curve was calculated (figure 17). There is an extremely high degree of nonspecific binding which was linear over the concentration range studied ($r = 0.99$). Subtraction of the nonspecific binding from the total amount of DA bound per mg protein produces a curve of specific binding (figure 18).

Scatchard analysis of this binding revealed a straight line ($r = 0.89$) indicating a single class of binding site and no cooperativity (figure 19). The intersection of the line at the ordinate indicated a B_{max} of approximately 39 fmols/mg protein with a K_D of 184 nM. Further analysis by the method of Hill (figure 20) produced a straight line ($r = 0.99$) with a slope of 1.03, consistent with first order mass action binding.

Figure 17

Binding of tritiated DA to crude membrane preparation. Binding in the absence (●) and presence (○) of 10 µM unlabelled DA was determined over the concentration range given. Each point represents the mean of triplicate samples from one representative experiment.

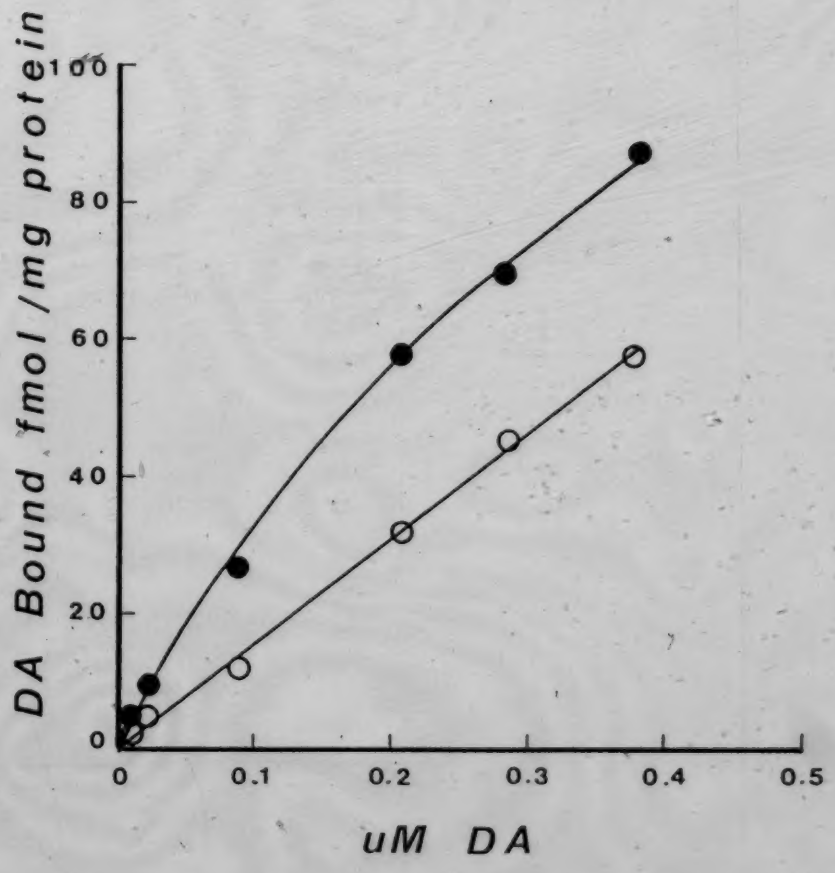

DA Bound fmol /mg protein
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
uM DA

Figure 18

Specific binding of labelled DA to crude membranes. The curve was obtained by subtraction of the nonspecific binding curve from the total binding curve in figure 17. Each point represents the mean values ± standard deviation from three experiments in triplicate.

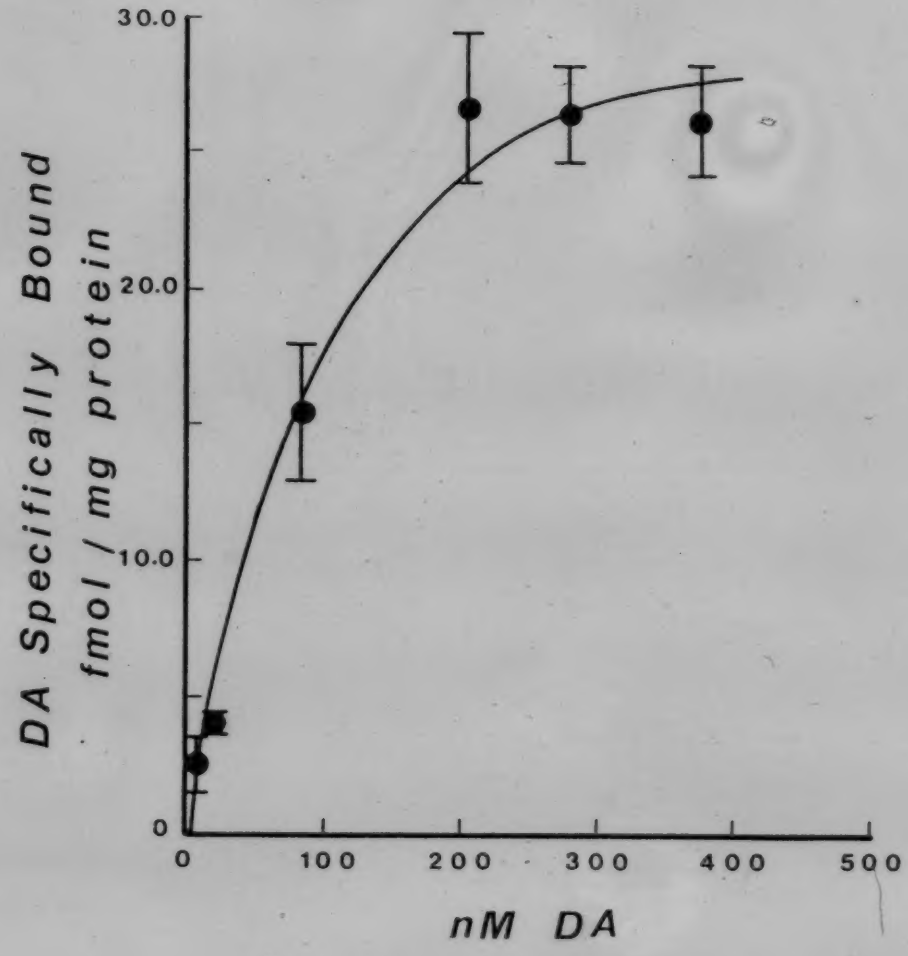

30.0
20.0
10.0
0
0
100
200
300
400
500
DA Specifically Bound
fmol / mg protein
nM DA

Figure 19

Scatchard analysis of binding. Each point represents the mean of triplicate samples from one representative experiment. The line displays a correlation of 0.89 with an apparent K_D of 184 nM and 39 fmols/mg protein B_{max}. B = fmols specifically bound DA; F = exogenous concentration of labelled DA.

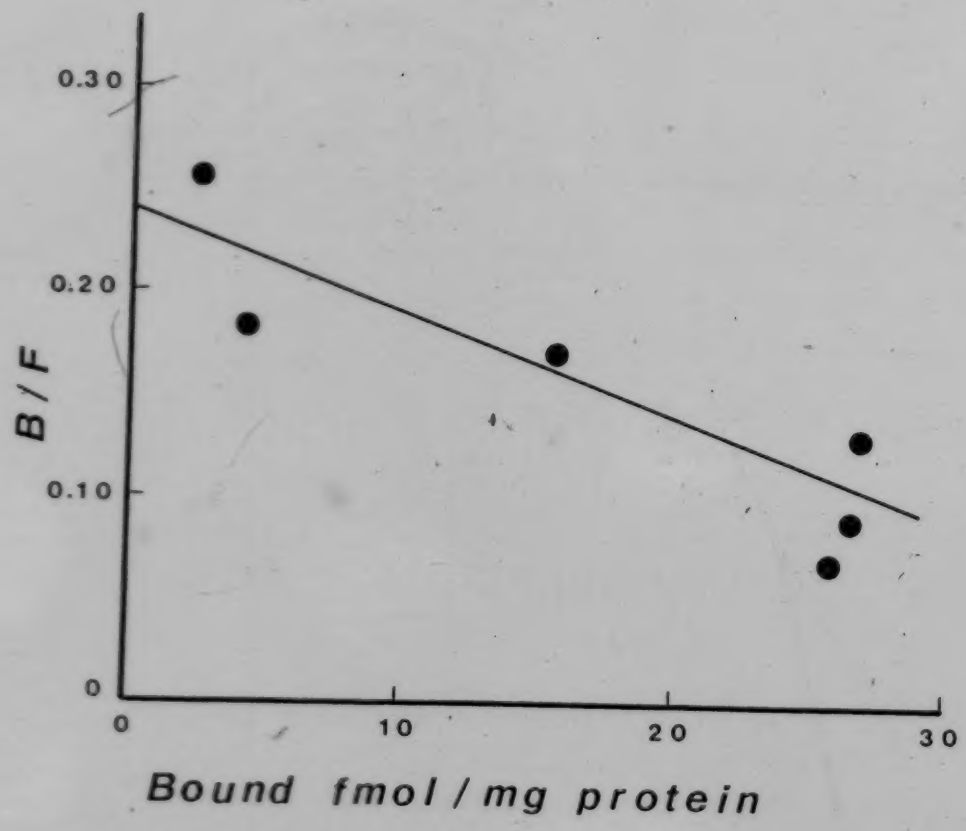
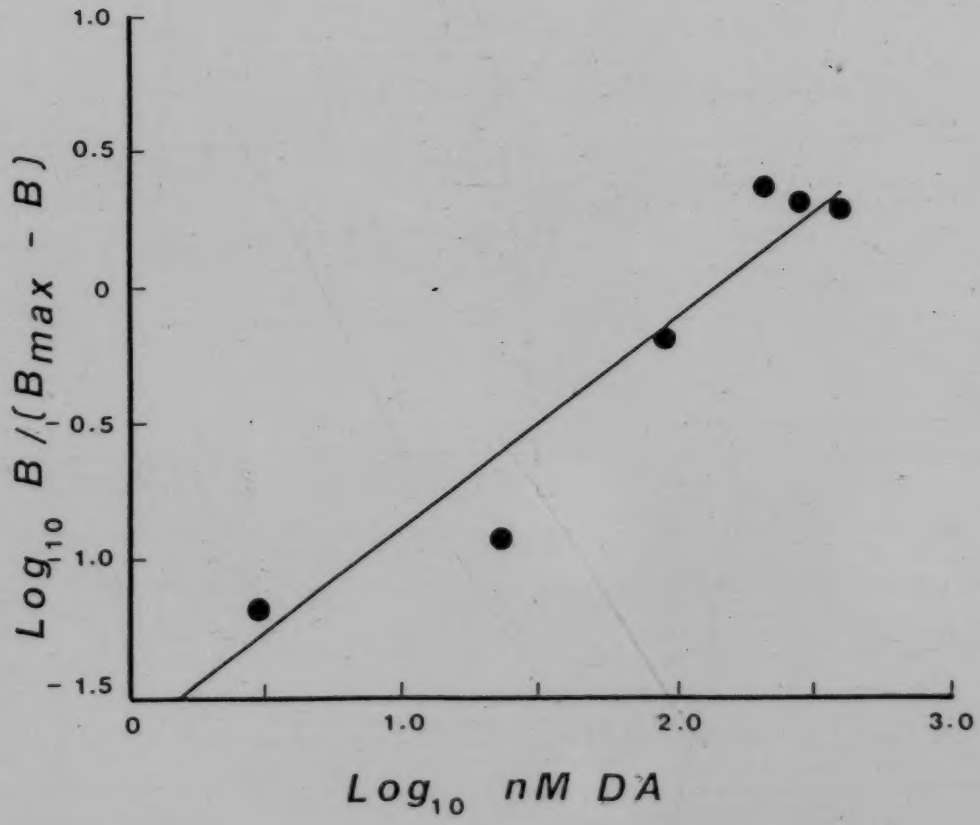

0.30
0.20
0.10
0
B/F
0
10
20
30
Bound fmol / mg protein

Figure 20

Hill analysis of binding. Analysis of the data using the Hill method revealed a slope of 1.03 ($r = 0.99$). Each point represents the mean of triplicate samples from one experiment. B = fmols specifically bound DA/ mg protein; B_{max} = maximum number of binding sites calculated using Scatchard analysis.


1.0
0.5
0
0.5
1.0
- 1.5
0
1.0
2.0
3.0
Log10 B /(Bmax - B)
Log10 nM DA

CHAPTER V

DISCUSSION

Previous studies of DA and 5-HT uptake by platelets, as briefly reviewed in Chapter II have described various aspects of the rate and the equilibrium state of the process of amine accumulation. Although the rate and equilibrium measurements provide useful information, each has it own limitations which must be recognized.

The uptake of DA and 5-HT can be regarded as consisting of a passive component and an energy-dependent component (Sneddon, 1973; Gordon and Olverman, 1978). The identification of these two uptake processes has proved difficult in practice. Gordon et al (1977) reported that the uptake rate measured within short incubation times (< 10 seconds) at low 5-HT concentration (< 10 μM) represents the energy requiring process. The authors also point out that at DA concentrations below the level required to saturate the energy-dependent process, passive diffusion contributes significantly to the total uptake.

At a state of uptake equilibrium, contributions of the passive and energy dependent components to the total amount of amine accumulated are distinct. The energy dependent component of uptake is estimated by subtracting the passive uptake measured in the presence of inhibitors of the energy dependent process from the total uptake. This introduces a source of error in the measurement. The uptake values reported in this study were measured at the state of uptake equilibrium (as defined in the methods section). Unlike the rate determination

which must be done at the steady state, the equilibrium state could be attained without strict time limitations. As shown in the results, characteristic responses of uptake to the inhibitors is demonstrated by the levels of accumulated amines at equilibrium.

Figures 5 and 6 show that addition of ammonium chloride to platelets in buffer solution causes efflux of accumulated amine. This phenomenon is observed whether ammonium chloride is added pre or post incubation. Such results are similar to those obtained by Ingebretsen and Flatmark (1979) with chromaffin granules of adrenal medulla. It is believed that the presence of ammonium ion facilitates the loss of internal protons, thus, dissipating the pH gradient (Chappel and Crofts, 1965). The excess extracellular ammonium ion drives ammonium ions aross the membrane. The hydrogen ions exit across the membrane concomitant with the influx of ammonium ion.

Drummond and Gordon (1975) reported evidence for three 5-HT binding sites in rat platelets. These researchers attributed transport to a middle affinity site. They arrived at a figure of approximately 700 nM for a dissociation constant for this site. These researchers also presented evidence for a third class of binding site of very low affinity (K_2 = 2μM). In human platelets, the third class of binding site has not been detected (Boullin et al, 1977). Previous kinetic analysis of 5-HT uptake indicated a Michaelis constant (Km) of approximately 1 μM (Gordon and Olverman, 1978). From these estimates, the ammonium chloride insensitive portion of the curve may be due to binding. The observation that ammonium chloride has no effect on binding supports this idea. However, we cannot rule out the possibility of a

sodium ion gradient (out > in) contributing to uptake. Ammonium chloride should collapse such an ion gradient. This point still remains obscure.

Accepting the idea that DA and 5-HT are partly accumulated by an energy dependent process, we must then determine if they are taken-up by the same mechanism. Experimental evidence points to the existence of a carrier for 5-HT uptake (Sneddon, 1973). This carrier, associated with the outer membrane, is sensitive to the tricyclic antidepressant imipramine, but not sensitive to reserpine (Rudnick, 1977). Imipramine competes with 5-HT at its middle affinity transport site (Drummond, 1976). Previous researchers postulated that should DA be taken up by an energy dependent process, or even facilitated diffusion, it would be mediated by the 5-HT carrier (Sneddon, 1973). Considering the evidence presented in figures 7 and 8, this may partly be the case. Imipramine is far more effective in blocking 5-HT uptake. DA uptake is inhibited approximately 15% by imipramine while 5-HT uptake is inhibited in excess of 80%.

Haldol, a DA antagonist (Creese et al, 1981) also displays differential inhibitory effects for the incorporation of these two monoamines. The concentrations of haldol required to produce 50% inhibition of uptake for DA and 5-HT are 0.18 and 5.60 µM, respectively (figure 11). Thus, haldol is approximately 30 fold more potent in blocking DA uptake as compared to 5-HT uptake. These results tend to concur with observations by past researchers. Trenchard et al (1975) using tricyclic transport inhibitors showed that DA uptake was not affected to the same extent as 5-HT uptake. Airaksinen et al (1980)

reported that modifications of functional group side chains on β-carbolines altered the inhibitor characteristics. The degree and specificity of inhibition was dependent upon the functional group alteration. Thus, depending on the group modification, an agent was found to inhibit DA and 5-HT uptake to different extents.

Earlier work by Boullin and O-Brien (1970) and Yamaguchi et al (1972) indicated that the total amount of DA accumulated over a 90-minute incubation period was abnormally low in platelets from Parkinsonian patients while the total amount of 5-HT taken up remained the same. These observations point to the possibility that the uptake mechanisms for DA and 5-HT in platelets are altered to different extents in Parkinson's disease. However, it is not clear whether the uptake mechanism is altered or that some other factors specific to DA accumulation are affected.

Gordon and Olverman (1978) reported that DA inhibited 5-HT uptake and vice versa. The inhibition constants reported correlated well with their respective Km's for uptake. The authors suggested that DA uptake is mediated by the carrier responsible for 5-HT uptake.

The binding of DA to platelet membranes revealed a K_D of 184 nM with approximately 39 fmol/mg protein binding sites. The extremely low number of binding sites makes it very difficult to determine, with great accuracy, the binding characteristics of DA to the crude membrane preparation. In fact, Tsai and Lefkowitz (1978) suggested that very low affinity binding sites are difficult to identify since specifically bound ligand is likely to be removed during the washing procedure. If

such is the case, the number of binding sites estimated by our study may fall short of the actual value.

This DA binding site does not appear to correspond to that found in the brain. The DA agonist DHEC has a K_D of approximately 2nM (Newman et al, 1978). Preliminary studies indicated that DHEC inhibits DA accumulation (data not shown). DHEC does not, however, have the potency that haldol has for inhibition of DA uptake. If we assume the site identified for DA binding is involved in the movement of DA across the platelet membrane, several lines of evidence fall into place. The relatively long equilibration period for DA uptake in comparison to that of 5-HT may be due to the difference in the number of binding sites for each ligand per cell. Preliminary studies for the binding of haldol to platelet membranes indicated a binding constant in the low µM range. This may explain the ability of haldol to abolish greater than 80% of DA accumulation. Haldol not only blocks DA uptake but also prevents the aggregatory response of the platelet to DA (Boullin et al, 1978). This is of interest since it lends credence to the possibility that haldol acts at the DA binding site purported by our binding studies.

Boullin et al (1978) indicated that neither haldol nor DA have binding sites on the platelet membrane. Their conclusions appear to be questionable on the following grounds: Binding experiments were carried out in PRP, resulting in high nonspecific binding. This is likely attributable to binding of ligand to plasma proteins. These authors measured the DA binding at concentrations up to 200 µM incubation for 3 minutes at 0°C. Figure 19 shows an approximate K_D of 184 nM. Since

only 6 points were plotted in their study, the concentration range appears too wide to facilitate proper analysis of a ligand-receptor interaction. Their experiments did indicate that haldol reduces the number of 5-HT binding sites. Thus, it is possible that haldol affects 5-HT and DA uptake by interfering with the binding of ligand to its respective carrier. It would seem from the data at hand that haldol has a higher affinity for the DA site than that of 5-HT.

In summary, the experimental evidence presented herein indicated that ammonium chloride suppresses a distinct part of DA and 5-HT uptake. 5-HT accumulation is more sensitive to imipramine while DA accumulation is more sensitive to haldol suggesting that the 5-HT carrier is different from the DA carrier. When crude membrane preparations from platelets were used to test the binding of DA, a single binding site could be detected. This binding site had an apparent Km of 184 nm and approximately 39 fmols/mg protein binding sites.

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